(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 September 2001 (13.09.2001)

PCT

(10) International Publication Number
WO 01/66598 A2

(51) International Patent Classification[7]: C07K 14/705, C12N 15/12, 15/10, 15/11, C12Q 1/68, C12N 15/66, 5/10, C07K 16/28, C12N 15/62, C07K 16/00, G01N 33/48

(21) International Application Number: PCT/US01/07073

(22) International Filing Date: 5 March 2001 (05.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/186,928 3 March 2000 (03.03.2000) US
60/187,231 3 March 2000 (03.03.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/66598 A2

(54) Title: CHEMOATTRACTANT RECEPTOR CHARACTERIZATION AND CELL SELECTION MATERIALS AND METHODS AND CHEMOKINE RECEPTOR CCR11 MATERIALS AND METHODS

(57) Abstract: The present invention provides novel polynucleotides encoding CCR11, a novel member of the G protein coupled receptor family, the novel polypeptide encoded by these polynucleotides and methods of making and using these and related products. The present invention also provides materials and methods for characterizing all receptors that mediate chemotaxis and compounds that stimulate chemotaxis. Cell lines produced by methods of the invention also are provided.

CHEMOATTRACTANT RECEPTOR CHARACTERIZATION AND CELL SELECTION MATERIALS AND METHODS AND CHEMOKINE RECEPTOR CCR11 MATERIALS AND METHODS

The present application claims priority benefit from United States Provisional Patent Application Numbers 60/186,928 and 60/187,231, both filed March 3, 2000, both of which are incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the fields of genetics and cellular and molecular biology. More particularly, the invention relates to a method of identifying cells that express chemoattractant receptors and cells that respond to chemoattractants, and also to novel chemoattractant G protein coupled receptor polynucleotides and polypeptides and uses thereof.

BACKGROUND

Intercellular communication is vital for the development and survival of multicellular organisms. Cell membrane receptor proteins expressed on the cell surface are among the molecules through which cells of an organism communicate with each other and obtain information and stimuli from their environment. Many such receptors have been identified, characterized, and classified into major receptor superfamilies based on structural motifs and signal transduction features. Such families include ligand-gated ion channel receptors, voltage-dependent ion channel receptors, receptor tyrosine kinases, receptor protein tyrosine phosphatases, and G protein-coupled receptors. The receptors are a first essential link for translating an extracellular signal into a cellular physiological response.

The G protein-coupled receptors (GPCRs) form a vast superfamily of cell surface receptors which are characterized by an amino-terminal extracellular domain and a carboxyl-terminal intracellular domain separated by amino acids forming a serpentine structure that passes through the cell membrane seven times. Hence, such receptors are sometimes also referred to as seven transmembrane (7TM)

receptors. These seven transmembrane domains define three extracellular loops which, like the amino-terminal domain, are accessible from outside the cell, and three intracellular loops which, like the carboxyl-terminal domain, are accessible from inside the cell. The extracellular portions of the receptor play a role in recognizing and binding one or more extracellular binding partners (ligands), whereas the intracellular portions have a role in interacting and communicating with downstream effector molecules. GPCR's have recently been reviewed in Premack and Schall, *Nat Med, 2*:1174-78 (1996); and Berger *et al.*, *Annu Rev Immunol, 17*: 657-700 (1999).

The GPCRs bind a variety of ligands including calcium ions, hormones, chemokines, neuropeptides, neurotransmitters, nucleotides, lipids and odorants, and even recognize photons. See generally S. K. Bohm *et al., Biochem J., 322*: 1-18 (1997). When a specific ligand binds to its corresponding receptor, the ligand stimulates the receptor to activate a specific heterotrimeric guanine-nucleotide-binding regulatory protein (G-protein) that is coupled to the intracellular portion of the receptor. The G protein in turn transmits a signal to an effector molecule within the cell, by either stimulating or inhibiting the activity of that effector molecule. These effector molecules include adenylate cyclase, phospholipases, and ion channels. Adenylate cyclase and phospholipases are enzymes that are involved in the production of the second messenger molecules cAMP, inositol triphosphate and diacylglycerol. It is through this sequence of events that an extracellular ligand stimuli exerts intracellular changes through a GPCR. Each such receptor has its own characteristic primary structure, expression pattern, ligand-binding profile, and intracellular effector system.

The importance of GPCRs as therapeutic targets cannot be overestimated. It is predicted that over one thousand GPCRs may exist, and several hundred have already been identified having a plethora of functions. Indeed, many currently approved and investigational drugs are directed to modulating GPCR activity and downstream physiological phenomena. Because such receptors have historically been so important as drug targets, much effort is expended to develop functional methods of identifying novel receptors, their ligands, and to elucidate the roles they play. See, *e.g.*, PCT International Publication WO 99/05274.

Of particular interest are G protein coupled receptors that bind chemokines. Chemokines are a family of small 8 to 10 kD proteins with 20 to 70 percent homology in amino acid sequences that are responsible for the directed migration of specific cell types. See Luster, A.D., *N Engl J Med 338*: 436-445 (1998). Chemokines induce cell migration and activation by binding to specific GPCRs on target cells.

Chemotaxis or chemotactic migration may be broadly defined as stimulated motility or orientation by cells that is directional with respect to a chemical stimulus. Alternatively, chemotaxis may be defined as the directional response of cells or organisms to chemical concentration gradients. Chemotaxis is differentiated from chemokinesis, in which the motion or orientation of the cells is non-directional or random. The chemical and biological substances that stimulate chemotaxis are known as chemoattractants; the cells that exhibit a chemotactic response sense the presence of those substances through receptors carried on their surfaces. The interactions between these receptors and their ligands and the consequences of those interactions are extraordinarily complex. Nonetheless, understanding this interplay is crucial to understanding the biology of multicellular organisms, as chemotaxis and chemotactic gradients are universally employed, in these organisms, from the laying down of embryonic tissues to the normal function of organs and systems in mature individuals.

Chemoattractants operative in the immune system are the subject of especially intense scrutiny within the medical community. The immune system is extraordinarily diverse in its structure and function, being diffuse throughout the body while also having foci organization in organs, such as lymphoid tissues, bone marrow, etc. Control and distribution of leukocytes, the principal cellular effector unit in this diverse system, is mediated through chemical and biological signals such as chemoattractants, as well as numerous other components including antibodies, the complement system, growth factors, and the like.

Chemoattractants include agonists of the fMLP receptor, such as fMet-Leu- Phe (fMLP) and other peptides blocked at the N-terminus by formylated methionine. Such peptides are not normally found in eukaryotes, but are

fundamentally characteristic of bacteria, and serve to signal the presence of bacteria to the immune system. Leukocytes that express the fMLP receptor, such as neutrophils and macrophages, migrate up gradients of these peptides toward loci of infection.

Another known chemoattractant is the leukotriene LTB_4, which is released following macrophage or mast cell activation at a site of inflammation. LTB_4 is generated by metabolism of arachidonic acid through the lipoxygenase pathway. In addition to its chemoattractant properties, LTB_4 also potentiates the activity of the prostaglandin PGE_2 in increasing vascular permeability. Neutrophils and macrophages each express receptors for LTB_4.

Another chemoattractant is C5a, which is released following complement activation. C5a receptors are found on neutrophils and macrophages, which are the principal cell types that exhibit a chemotactic response to the molecule. This molecule has numerous other functions, including mediating mast cell degranulation, neutrophil activation, smooth muscle contraction, and increased capillary permeability. Other chemoattractant molecules generated through the blood clotting system include fibrin peptide B (fibrinopeptide B), and thrombin, which attract phagocytes.

By far the largest group of chemoattractants, however, is a family of small proteins known as chemokines. The chemokines are a rapidly expanding superfamily of molecules that have recently been the focus of increasing interest. For reviews see Zlotnik, *et al.*, *Critical Reviews in Immunology, 19*: 1-47 (1999); Baggiolini et al., *Annu Rev Immunol 15*: 675-705 (1997); Rollins, *Blood, 90*: 909-28 (1997); Schall and Bacon, *Curr Opin Immunol, 6*: 865-73 (1994); and Luster, *N Engl J Med, 338*: 436-45 (1998). This superfamily is subdivided into families on the basis of the relative position of cysteine residues in the mature chemokine protein. Two major families were known before 1993, the alpha or CXC family and the beta or CC family. In the alpha chemokines, the first two cysteine residues are separated by a single amino acid (CXC), whereas in the beta chemokines, the first two cysteine residues are adjacent to each other (CC).

The CXC chemokines can be further divided into two subclasses based upon the presence or absence of an ELR amino acid motif immediately preceding

Cys-1 (ELR$^+$). This motif confers the ability to bind to CXCR2, and members of this largest group of CXC chemokines are generally neutrophil chemoattractants. The CXC chemokines that lack the ELR motif (ELR$^-$) are primarily lymphocyte chemoattractants and bind to one of the GPCRs: CXCR3 (Mig, IP-10, I-TAC), CXCR4 (SDF-1), or CXCR5 (BLC-1/BCA-1).

Two other classes of chemokines have been described since 1993. The gamma class lacks the first and third cysteines and is represented by lymphotactin. The delta class is the CX_3C type that exhibits three amino acids between the first two cysteines. Fractalkine and neurotactin are examples of CX_3C chemokines.

The CC chemokines, in general, do not act on neutrophils but attract monocytes, eosinophils, basophils, and lymphocytes. Structurally, the CC chemokines can be subdivided into two families, the monocyte-chemoattractant-protein-eotaxin family (MCP), containing all five monocyte chemoattractant proteins (MCP-1, MCP-2, MCP-3, MCP-4 and MCP-5) and eotaxin, which are approximately 65 percent identical to each other, and a second family comprising all other CC chemokines. The MCP chemokines can be further divided into two functional groups based upon whether they bind to CCR2 and attract primarily monocytes and lymphocytes or to CCR3 and attract primarily eosinophils and basophils. Some members of the MCP family bind to both receptors and exhibit a corresponding spectrum of biological activity.

MCP-4, first described by Uguccioni, *et al., J. Exp. Med., 185*: 2379-2384 (1996), is structurally a typical CC chemokine of the MCP subfamily and shares approximately 60% amino acid identity with MCP-1, MCP-3 and eotaxin. MCP-4 appears to be widely expressed in a variety of tissue types, including small intestine and colon, where expression is the highest, and also in thymus, lung, placenta and heart as reported in Godiska, *et al, J. Leukoc. Biol., 61(3)*: 353-360 (1997). As might be expected, considering its relationship to the MCP subfamily, MCP-4 shows potent monocyte- and lymphocyte-attracting activity. The more striking chemotactic function of MCP-4, however, is its ability to attract eosinophils, which rivals that of eotaxin as described in Garcia-Zepeda, *et al., J. Immunol., 157(12)*: 5613-5622 (1996).

Several receptors for MCP-4 have been identified, including the GPCRs CCR2 and CCR3. Zlotnik, *supra*. Zlotnik postulates that the lymphocyte-attracting functions of MCP-4 are mediated by binding to GPCR CCR2, while the eotaxin/basophil activities of MCP-4 are likely to be mediated primarily by binding to GPCR CCR3. While MCP-4 and eotaxin have many properties in common and appear to be functional equivalents, Uguccioni, *et al., J. Clin. Invest., 100(5)*:1137-1143 (1997) disclosed that an anti-CCR3 antibody that blocked eotaxin-mediated effects on basophils only partially inhibited the effects of MCP-4. It seems therefore that MCP-4 effects on basophils can be mediated by another receptor or that MCP-4 binds to GPCR CCR3 in a slightly different manner than eotaxin.

Chemokines and the GPCRs that recognize them play a critical role in the host response to infection since they are responsible for recruitment of leukocyte subsets to sites of pathogen entry. See, *e.g.*, Gerard, *Nature, 395*: 217-219 (1998); Baggiolini, *et al., Nature, 392*: 565-568 (1998). In addition, many inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma have been associated with elevated chemokine expression. Luster *supra;* Wells and Proudfoot, *Inflamm Res, 48*: 353-362 (1999). Furthermore, chemokines are also responsible for the migration of cells within certain lymphoid organs, such as thymus [Suzuki *et al., J Immunol, 162*: 5981-85 (1999); Campbell *et al., J Immunol, 163*: 2353-57 (1999); Chantry *et al., Blood, 94*: 1890-1898 (1999)] lymph node [Cyster *et al., Curr Top Microbiol Immunol, 246*: 87-92 (1999)] and spleen [Pevzner *et al., Curr Top Microbiol Immunol, 246*: 79-84 (1999)], that are critical for leukocyte development. For a general review of the role of chemokines in cellular migration in lymphoid organs, see, Cyster, *Science, 286*: 2098-102 (1999). Pevzner, *et al., Curr. Top. Microbiol. Immunol., 246*: 79-84 (1998) confirms that chemokine receptors play an essential role as conductors of an orchestra of adhesion molecules which allow lymphocyte subsets to navigate to different anatomical locations and are involved in the functional compartmentalization of lymphoid organs.

Chemokines have also been implicated in cardiovascular processes such as angiogenesis and atherosclerosis. Specifically, Gosling, *et al., J Clin Invest,*

103: 773-778 (1999) demonstrated that MCP-1, a CC chemokine, plays a critical role in the initiation of atherosclerosis.

Five human CXC chemokine receptors, CXCR1 through CXCR5, nine human CC chemokine receptors, CCR1-CCR9, and one human CX_3C chemokine receptor, CX_3CR1, have been identified. Chemokine receptors are expressed on different types of leukocytes. Some receptors are restricted to certain cells, *e.g.*, CXCR1 is predominately restricted to neutrophils, whereas others are more widely expressed, *e.g.*, CCR2 is expressed on monocytes, T-cells, natural killer cells, dendritic cells, and basophils. In addition, chemokine receptors can be constitutively or inducibly expressed depending upon the cell type. For example, CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes only after stimulation by interleukin-2. Loetscher, *et al.*, *J. Exp. Med.*, *184*: 569-577 (1996). Although most chemokine receptors bind more than one chemokine, CC receptors bind only CC chemokines and CXC receptors bind only CXC chemokines.

Because of the vital role of GPCRs in the communication between cells and their environment, such receptors are attractive targets for therapeutic intervention. There thus remains a need in the art for identifying additional G protein coupled receptors that bind chemokines and for further characterizing their structure and function.

Previously characterized GPCRs that function as chemokine receptors do share significant homology with each other, having 25-65% identical amino acids. As a group, the chemokine receptors are organized as a branch of the GPCR family tree. Many chemokine receptors were originally identified as orphan GPCRs. There remain several orphan GPCRs with high similarity to the chemokine receptor family, but for which chemokine ligands have not been identified. Accordingly, these putative receptors cannot yet be categorized as chemokine receptors.

The expression of cloned receptor proteins, such as GPCRs, at high levels in cell lines is widely used within the pharmaceutical industry to identify receptor- specific agonists or antagonists. In addition to providing the basis for high

throughput screens, such cell lines are also used in secondary assays to test the biological efficacy and receptor specificity of candidate modulating compounds.

Current methods to identify transfectants that express the receptor of interest include northern blotting (to show expression of the transgene), antibody staining (which is dependent upon availability of a receptor-specific antibody or requires "epitope-tagging" of the receptor which may affect ligand specificity), or direct binding studies on lines or clones of cells. These methods are labor intensive and do not permit direct selection of cells that express recombinant biologically active receptors.

One of the characteristic features of chemokines is their ability to induce chemotaxis or migration by cells expressing functional chemokine receptors. A method has been described for obtaining a cell population enriched for cells expressing elevated levels of a recombinant receptor from a mixed cell population expressing various levels of the receptor [Tiffany *et al.*, *J Exp Med, 186*: 165-70 (1997)]. As described, this method is directed to isolation of a cell type that is known beforehand to express a particular transfected receptor that is known to be biologically active. Selection of this receptor is accomplished by using particular agonists to which it is known to bind. The receptor-expressing cells are enriched by collecting those cells that migrate toward the chemokine in a chemotaxis apparatus. As described, clones are then obtained by limiting dilution, and receptor expression is confirmed by flow cytometry.

It is recognized in the art that identification of ligands for orphan chemoattractant receptors, *e.g.*, orphan GPCRs can be complicated. Such receptors can exhibit paradoxical behavior, particularly transfected recombinant receptors. While not well understood, such unusual behavior may be due to overexpression of recombinant receptors, inappropriate intracellular signaling protein usage, or other yet unidentified phenomena. In addition, our laboratory has noted that some chemokine receptors are not expressed in a stable fashion and that functional responses can be lost if not repeatedly selected for. Overexpression is a natural consequence of using a strong viral promoter such as CMV, and may lead to functional responses that are potentially deleterious to transfected cells. Some changes we have observed with

receptor transfectants include increases in cell adhesiveness or decrease in growth rate.

Consequently, assessment of orphan receptors to identify them as functional chemoattractant receptors is typically laborious and time-consuming. This is particularly true of systems in which functional receptor is expressed in only a small subset of the transfected cells. For example, if an expressed receptor is functional in only 0.01% of cells, then a functional assay to determine calcium flux may give false negative results. Analysis of individual clones from such bulk populations would require examination of an average of >10,000 clones to identify a cell population that expresses a functional receptor. In our experience, identification of functional receptors using such methods is typically fortuitous.

In view of the above considerations, it is clear that existing methods for characterizing orphan receptors are profoundly limited by technical considerations. Furthermore, methods of identifying novel ligands for known chemoattractant receptors and methods of identifying and isolating cells expressing chemotaxis-mediating receptors are neither as practical nor as predictable as desired.

SUMMARY OF THE INVENTION

The present invention provides materials and methods that address one or more of the above-described needs.

For example, the present invention provides materials and methods that represent novel tools for manipulating G protein coupled receptors and other chemoattractant receptors, which are valuable tools for, e.g., characterizing orphan chemoattractant receptors and orphan chemoattractant molecules. Thus, in one aspect, the invention provides a method for screening test compounds to identify novel ligands for chemoattractant receptors. The invention also provides a method for screening receptors to identify novel chemoattractant functions for such receptors. The invention further provides direct methods for identifying and isolating cells that express functional chemoattractant receptors. The invention provides a simple and effective method for screening orphan receptors to identify functional features of the receptors based on ligand interactions.

In one aspect, the invention provides a method of screening cells modified to express a cell surface protein, wherein said protein is not known to mediate chemotaxis. For example, the invention provides a method comprising:

(a) contacting the cells expressing the functionally uncharacterized protein with a test compound; and

(b) detecting chemotactic migration by the cells in response to the test compound,

whereby chemotactic migration by the cells indicates that the cells express the protein as a functional receptor that mediates chemotaxis in response to the test compound and/or that the test compound is a chemoattractant ligand for the protein.

The test compound can be a known chemoattractant, such as a known chemokine, including a known chemokine that mediates chemotaxis through another, known chemoattractant receptor. Alternatively, the test compound may not have previously been identified as having chemoattractant properties, *i.e.*, wherein the test compound is not known to mediate chemotaxis through a known chemoattractant receptor. The test compound may be selected from the group consisting of chemokines, chemotactic lipids, chemotactic peptides, and functional analogs thereof. The protein may be an orphan G-protein coupled receptor whose function is uncharacterized with respect to mediation of chemotaxis. The method may further comprise steps of:

(c) isolating a cell that exhibits chemotactic migration in step (b); and

(d) culturing the cell in a medium that supports growth and proliferation of the cell;

whereby the method provides a clonal population of cells expressing the protein as a functional receptor that mediates chemotaxis in response to the test compound.

Accordingly, the invention also provides a cell culture comprising cells that have been cultured according to the described method.

In another aspect, the invention provides a method of screening cells expressing a chemoattractant receptor. For example, the invention provides a method comprising:

(a) contacting the cells with a test compound, wherein said test compound is not known to elicit chemotaxis through the receptor; and

(b) detecting chemotactic migration by the cells in response to the test compound,

whereby chemotactic migration by the cells indicates that the cells express the receptor as a functional receptor that mediates chemotaxis in response to the test compound and/or that the test compound is a chemoattractant ligand for the receptor.

The receptor may be a G-protein coupled receptor (GPCR). Moreover, the receptor may not be known beforehand to mediate chemotaxis in response to a known chemoattractant. Alternatively, the receptor may be known to mediate chemotaxis in response to a known chemoattractant, such as a chemoattractant selected from the group consisting of chemokines, chemotactic lipids, and chemotactic peptides, and functional analogs thereof. The method may further comprise steps of:

(c) isolating a cell that exhibits chemotactic migration in step (b); and

(d) culturing the cell in a medium that supports growth and proliferation of the cell;

whereby the method provides a clonal population of cells expressing the protein as a functional receptor that mediates chemotaxis in response to the test compound. Cell cultures comprising cells that have been cultured according to the method are contemplated under the invention.

In another aspect, the invention provides a method of deriving a clonal population of cells expressing a functional chemoattractant receptor. For example, in this aspect, the method comprises:

(a) providing a cell population comprising cells that express a chemoattractant receptor of interest;

(b) contacting the cell population with a chemotaxis-inducing amount of a test compound that is a functional ligand of the receptor, under conditions that permit chemotaxis by functional receptor-expressing cells in response to stimulus by the test compound;

(c) collecting a responsive cell subset comprising cells that exhibit chemotaxis in the presence of the test compound; and

(d) culturing an isolated cell from the responsive cell subset in a medium that supports growth and proliferation of the isolated cell,

whereby there is established a clonal population of functional receptor-expressing cells in culture. Thus, there may be provided a cell or cells expressing functional chemoattractant receptor, isolable from cells transfected by an expression vector containing DNA encoding the receptor by means of the described method.

In another aspect, the invention provides a method of isolating cells expressing a chemoattractant receptor of interest. For example, in one embodiment, the invention provides a method of isolating cells expressing a chemoattractant receptor of interest, comprising steps of:

(a) providing a reservoir comprising a cell-permeable membrane separating a cell chamber and a ligand chamber in the reservoir, wherein the cell chamber contains receptor-expressing cells in a cell medium;

(b) adding a ligand composition to the ligand chamber of the reservoir and permitting the ligand composition to contact the cell-permeable membrane;

(c) incubating the cells for a time sufficient to allow chemotactic migration of cells that are chemotactic to the composition across the cell-permeable membrane; and

(d) collecting cells that migrated across the cell-permeable membrane during the incubating step, thereby providing a cell population enriched for cells that express a functional receptor for a chemoattractant that is present in the composition. Such cells can be further cultured and cloned, and, optionally, subjected to additional rounds of isolation according to steps (a)-(d). The ligand composition comprises any test compound of interest, especially compounds known or suspected of having chemotactic effects on the cells.

In a related embodiment, the invention provides a method that comprises:

(a) providing a cell reservoir having a bottom comprising a cell permeable membrane having upper and lower surfaces, wherein the cell reservoir contains receptor-expressing cells in a medium that is substantially free of agents that induce

chemotaxis mediated by the receptor, and wherein the medium is in contact with the upper surface of the cell permeable membrane;

(b) providing a ligand reservoir containing a test medium comprising a test compound;

(c) contacting the cell reservoir with the ligand reservoir whereby the cell permeable membrane is placed in contact with the test medium;

(d) incubating the cells for a time sufficient to allow chemotactic migration of cells across the cell permeable membrane in response to the test compound; and

(e) collecting cells that migrate across the cell permeable membrane into the ligand reservoir,

whereby there is provided a cell population enriched for cells that express functional chemoattractant receptor that mediates chemotactic migration in response to the test compound.

The method may further comprise the steps of:

(f) cloning a cell collected in step e); and

(g) culturing the cloned cell in a medium that supports growth and proliferation of the cell,

whereby there is provided a substantially homogeneous cell population consisting essentially of cells that migrate in response to the test compound.

As noted, the methods of the invention may be used to define novel chemoattractant receptor/ligand pairs in which a previously unknown relationship between a receptor and a ligand is established. Thus, the method may be used to establish new ligands for a known receptor or to establish that a known chemoattractant binds to other previously unrecognized receptors. The methods of the invention may in fact be used to establish a functional relationship between a receptor and a ligand wherein neither has previously been recognized to mediate chemotaxis.

Accordingly, in the methods of the invention, the receptor is optionally a protein whose capacity to mediate chemotaxis in previously unknown or uncharacterized. Alternatively, the receptor is a protein previously recognized to mediate chemotaxis, but whose capacity to mediate chemotaxis is uncharacterized

with respect to the test compound. In one variation, the test compound is a compound that is uncharacterized as to its capacity to induce chemotaxis in cells. Alternatively, the test compound may be known to have some chemotactic potency in other systems, but uncharacterized as to its capacity to induce chemotaxis in cells expressing the receptor of interest.

Materials and methods useful for characterization of chemokine receptors and preparation of chemokine receptor-expressing cell lines are described in greater detail below.

The present invention also provides specific chemokine receptor materials and methods. For example, the present invention provides purified and isolated polynucleotides encoding CCR11 polypeptides. The invention includes both naturally occurring and non-naturally occurring CCR11-encoding polynucleotides. Naturally occurring polynucleotides of the invention include, for example, allelic and splice variants, as well as species homologs (or orthologs) expressed in cells of different animals. Non-naturally occurring CCR11 encoding polynucleotides include analogs or variants of the naturally occurring products, such as insertion variants, deletion variants, substitution variants, and derivatives, as described below. The polynucleotides of the present invention still further include, but are not limited to, a polynucleotide comprising the nucleotide sequence of the cDNA insert of clone p1 21679 deposited on March 2, 2000 under Accession No. PTA-1439 with the American Type Culture Collection (ATCC; 10801 University Blvd., Manassas, Virginia, 20110-2209, USA) or a CCR11 protein coding portion thereof, such as the full length protein coding sequence. In a preferred embodiment, the invention provides a polynucleotide comprising the sequence set forth in SEQ ID NO: 1. The invention also embraces polynucleotides encoding the amino acid sequence set out in SEQ ID NO: 2. A presently preferred polypeptide of the invention comprises the amino acid sequence set out in SEQ ID NO: 2. Anti-sense polynucleotides are also provided.

The invention also provides expression constructs (or vectors) comprising polynucleotides of the invention, and host cells comprising a polynucleotide or an expression construct of the invention. Methods to produce a

polypeptide of the invention are also comprehended. The invention further provides antibodies, preferably monoclonal antibodies, specifically immunoreactive with a polypeptide of the invention, as well as hybridomas that secrete the antibodies.

The invention also provides CCR11 polypeptides encoded by a polynucleotide of the invention. CCR11 polypeptides can be naturally or non-naturally occurring. The invention further provides binding partner compounds (ligands) that interact with a CCR11 polypeptide of the invention. Methods to identify ligands are also provided, as well as methods to identify modulators of CCR11 polypeptides.

The invention also provides compositions, and preferably pharmaceutical compositions, comprising a CCR11 polypeptide, a CCR11 antibody, other CCR11 modulators, or a combination of these compounds. When compositions of the invention, and in particular pharmaceutical compositions, are used for therapeutic or prophylactic intervention, the compounds can include one or more pharmaceutically acceptable carriers. Methods of packaging a composition of the invention, as well as methods for delivery and therapeutic treatment are also provided.

Thus, in one aspect, the invention provides novel purified and isolated human polynucleotides (*e.g.*, DNA sequences and RNA transcripts, both sense and complementary anti-sense strands) encoding the human CCR11 polypeptides. DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. Genomic DNA of the invention comprises the protein coding region for a polypeptide of the invention and includes allelic variants of the preferred polynucleotide of the invention. Allelic variants are known in the art to be modified forms of a wild type gene sequence, the modification resulting from recombination during chromosomal segregation or exposure to conditions which give rise to genetic mutation. Allelic variants, like wild type genes, are inherently naturally occurring sequences and can be isolated from different organisms of the same species (as opposed to non-naturally occurring variants which arise from *in vitro* manipulation). Splice variants are also comprehended by the invention.

The invention comprehends cDNA that is obtained through reverse transcription of an RNA polynucleotide encoding CCR11, followed by second strand synthesis of a complementary strand to provide a double stranded DNA.

"Chemically synthesized" as used herein and understood in the art, refers to polynucleotides produced by purely chemical, as opposed to enzymatic, methods. "Wholly" chemically synthesized DNA sequences are therefore produced entirely by chemical means, and "partially" synthesized DNAs embrace those wherein only portions of the resulting DNA were produced by chemical means.

A preferred DNA sequence encoding a human CCR11 polypeptide is set out in SEQ ID NO: 1. The worker of skill in the art will readily appreciate that the preferred DNA of the invention comprises a double stranded molecule, for example, the molecule having the sequence set forth in SEQ ID NO: 1 along with the complementary molecule (the "non-coding strand" or "complement") having a sequence deducible from the sequence of SEQ ID NO: 1 according to Watson-Crick base pairing rules for DNA. Single stranded polynucleotides, RNA as well as coding and noncoding DNAs, are also embraced the invention. Also preferred are polynucleotides encoding the CCR11 polypeptide of SEQ ID NO: 2.

The invention further embraces CCR11 DNAs isolated from other species, preferably mammals, which share sequence identity with human CCR11 DNA. Percent sequence "identity" with respect to polynucleotides of the invention is defined herein as the percentage of nucleotide bases in the candidate sequence that are identical to nucleotides in the CCR11 sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

The polynucleotide sequence information provided by the invention makes possible large scale expression of the encoded CCR11 polypeptide by techniques well known and routinely practiced in the art. Polynucleotides of the invention also permit identification and isolation of polynucleotides encoding related CCR11 polypeptides by well known techniques including Southern and/or Northern hybridization, polymerase chain reaction (PCR), and variations of PCR. Examples of related polynucleotides are polynucleotides encoding polypeptides homologous to

CCR11 and structurally related polypeptides sharing one or more properties of CCR11.

Properties of CCR11 include, for example, its interaction with chemokine ligands (e.g., ELC (EBII Ligand Chemokine, also known as MIP-3β, Genbank Acc. No. Q99731), SLC (Secondary Lymphoid tissue Chemokine, GENBANK Acc. No. O00585), TECK (Thymus-Exporessed ChemoKine, Genbank Acc. No. O15444)), immunological properties, interaction with G protein(s), and seven hydrophobic transmembrane domains, as well as the ability to cause cell migration towards chemokine ligands.

The disclosure of a full length polynucleotide encoding a CCR11 polypeptide makes readily available to the worker of ordinary skill in the art every possible fragment of the full length polynucleotide. The invention therefore provides fragments of CCR11-encoding polynucleotides comprising at least 10 to 20, and preferably at least 15, consecutive nucleotides of a polynucleotide encoding CCR11. Preferably, fragment polynucleotides of the invention comprise sequences unique to the CCR11-encoding polynucleotide, and therefore hybridize under highly stringent or moderately stringent conditions only (*i.e.*, "specifically" or "exclusively") to polynucleotides encoding CCR11, or CCR11 fragments thereof, containing the unique sequence. Polynucleotide fragments of genomic sequences of the invention comprise not only sequences unique to the coding region, but also include fragments of the full length sequence derived from introns, regulatory regions, and/or other non-translated sequences. Sequences unique to polynucleotides of the invention are recognizable through sequence comparison to other known polynucleotides, and can be identified through use of alignment programs routinely utilized in the art, *e.g.*, those made available from public sequence databases.

Polynucleotide fragments of the invention can be labeled in a manner that permits their detection, including radioactive and non-radioactive labeling. Labeled fragment polynucleotides are particularly useful as probes for detection of full length or other fragment CCR11 polynucleotides. One or more fragment polynucleotides can be included in kits that are used to detect the presence of a polynucleotide encoding CCR11, or used to detect variations in a polynucleotide

sequence encoding CCR11, including polymorphisms, and particularly single nucleotide polymorphisms. Kits of the invention optionally include a container and/or a label.

The invention also embraces naturally or non-naturally occurring CCR11-encoding polynucleotides that are fused, or ligated, to a heterologous polynucleotide to encode a fusion (or chimeric) protein comprising all or part of a CCR11 polypeptide. "Heterologous" polynucleotides include sequences that are not found adjacent, or as part of, CCR11-encoding sequences in nature. The heterologous polynucleotide sequence can be separated from the CCR11-coding sequence by an encoded cleavage site that will permit removal of non-CCR11 polypeptide sequences from the expressed fusion protein. Heterologous polynucleotide sequences can include those encoding epitopes, such as poly-histidine sequences, FLAG® tags, glutathione-S-transferase, green fluorescent protein, thioredoxin, and/or maltose binding protein domains, that facilitate purification of the fusion protein; those encoding domains, such as leucine zipper motifs, that promote multimer formation between the fusion protein and itself or other proteins; and those encoding immunoglobulins or fragments thereof that can enhance circulatory half-life of the encoded protein.

The invention also embraces DNA sequences encoding CCR11 polypeptides that hybridize under highly or moderately stringent conditions to the non-coding strand, or complement, of the polynucleotide in SEQ ID NO: 1. CCR11-encoding polynucleotides of the invention include (a) the polynucleotide set out in SEQ ID NO: 1; (b) polynucleotides encoding a polypeptide encoded by the polynucleotide of (a), and c) polynucleotides that hybridize to the complement of the polynucleotides of (a) or (b) under moderate or high stringency conditions. Exemplary high stringency conditions include a final wash in 0.2X SSC/0.1% SDS at 50°C to 65°C, and exemplary moderate stringency conditions include a final wash at 2X SSC/0.1% SDS at 42-50°C. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described in Ausubel, *et al.* (Eds.), *Protocols in Molecular Biology*, John Wiley & Sons, New York, New York (1994), pp. 6.0.3 to 6.4.10.

Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, *et al.*, (Eds.), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51.

CCR11 identified by hybridization, in general, share at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity with SEQ ID NO: 1.

Autonomously replicating recombinant expression constructs such as plasmid and viral DNA vectors incorporating CCR11-encoding sequences are also provided. Expression constructs wherein CCR11-encoding polynucleotides are operatively linked to an endogenous or exogenous expression control DNA sequence and a transcription terminator are also provided. Expression control DNA sequences include promoters, enhancers, and/or operators, and are generally selected based on the expression systems in which the expression construct is to be utilized. Preferred promoter and enhancer sequences are generally selected for the ability to increase gene expression, while operator sequences are generally selected for the ability to regulate gene expression. It is understood in the art that the choice of host cell is relevant to selection of an appropriate regulatory sequence. Expression constructs of the invention may also include sequences encoding one or more selectable markers that permit identification of host cells bearing the construct. Expression constructs may also include sequences that facilitate, and preferably promote, homologous recombination in a host cell. Preferred constructs of the invention also include sequences necessary for replication in a host cell.

Expression constructs are preferably utilized for production of an encoded protein, but may also be utilized to amplify the construct itself when other amplification techniques are impractical.

According to another aspect of the invention, host cells are provided, including prokaryotic and eukaryotic cells, comprising a polynucleotide of the invention in a manner which permits expression of the encoded CCR11 polypeptide. Polynucleotides of the invention may be introduced into the host cell as part of a

circular plasmid, as linear DNA comprising an isolated protein coding region or as part of a viral vector. Methods for introducing DNA into the host cell well known and routinely practiced in the art (*e.g.*, transformation, transfection, electroporation, nuclear injection, or fusion with carriers such as liposomes, micelles, ghost cells, protoplasts, and other transformed cells). Expression systems of the invention include bacterial, yeast, fungal, plant, insect, invertebrate, and mammalian cell systems.

Host cells of the invention are a valuable source of immunogen for development of antibodies specifically (*i.e.*, exclusively) immunoreactive with CCR11. Host cells of the invention are also useful in methods for large scale production of CCR11 polypeptides wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated from the cells or from the medium in which the cells are grown by purification methods known in the art (*e.g.*, conventional chromatographic methods including immunoaffinity chromatography, receptor affinity chromatography, hydrophobic interaction chromatography, lectin affinity chromatography, size exclusion filtration, cation or anion exchange chromatography, high pressure liquid chromatography (HPLC), reverse phase HPLC, and the like). Still other methods of purification include those wherein the desired protein is expressed and purified as a fusion protein having a specific tag, label, or chelating moiety that is recognized by a specific binding partner or agent. The purified protein can be cleaved to yield the desired protein, or be left as an intact fusion protein. Cleavage of the fusion component may produce a form of the desired protein having additional amino acid residues as a result of the cleavage process.

Knowledge of CCR11-encoding DNA sequences allows for modification of cells to permit, or increase, expression of endogenous CCR11. Cells can be modified (*e.g.*, by homologous recombination) to provide increased CCR11 expression by replacing, in whole or in part, the naturally occurring CCR11 promoter with all or part of a heterologous promoter so that the cells express CCR11 at higher levels. The heterologous promoter is inserted in such a manner that it is operatively linked to CCR11-encoding sequences. See, for example, PCT International Publication No. WO 94/12650, PCT International Publication No. WO 92/20808, and PCT International Publication No. WO 91/09955. It is also contemplated that, in

addition to heterologous promoter DNA, amplifiable marker DNA (*e.g.*, ada, dhfr, and the multifunctional CAD gene which encodes carbamyl phosphate synthase, aspartate transcarbamylase, and dihydroorotase) and/or intron DNA may be inserted along with the heterologous promoter DNA. If linked to the CCR11 coding sequence, amplification of the marker DNA by standard selection methods results in co-amplification of the CCR11 coding sequences in the cells.

The DNA sequence information provided by the present invention also makes possible the development through, *e.g.* homologous recombination or "knock-out" strategies Capecchi, *Science, 244*: 1288-1292 (1989), of animals that fail to express functional CCR11 or that express a variant of CCR11. Such animals are useful as models for studying the *in vivo* activities of CCR11 and modulators of CCR11.

The invention also provides purified and isolated mammalian CCR11 polypeptides encoded by a polynucleotide of the invention. Presently preferred is a human CCR11 polypeptide comprising the amino acid sequence set out in SEQ ID NO: 2. The invention also embraces CCR11 polypeptides encoded by a DNA selected from the group consisting of: (a) the polynucleotide set out in SEQ ID NO: 1; (b) polynucleotides encoding a polypeptide encoded by the polynucleotide of (a), and c) polynucleotides that hybridize to the complement of the polynucleotides of (a) or (b) under moderate or high stringency conditions.

The invention also embraces polypeptides have at least 99%,at least 95%, at least 90%, or at least 87% identity and/or homology to the preferred polypeptide of the invention. Percent amino acid sequence "identity" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with residues in the CCR11 sequence after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent sequence "homology" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the CCR11 sequence after aligning the sequences and introducing gaps, if

necessary, to achieve the maximum percent sequence identity, and also considering any conservative substitutions as part of the sequence identity.

Polypeptides of the invention may be isolated from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving host cells of the invention. Use of mammalian host cells is expected to provide for such post-translational modifications (*e.g.*, glycosylation, truncation, lipidation, and phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Glycosylated and non-glycosylated forms of CCR11 polypeptides are embraced.

Polypeptides of the invention include variant (or analog) CCR11 polypeptides.

In one example, insertion variants are provided wherein one or more amino acid residues supplement a CCR11 amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the CCR11 amino acid sequence. Insertional variants with additional residues at either or both termini can include for example, fusion proteins and proteins including amino acid tags or labels. Insertion variants may be CCR11 polypeptides wherein one or more amino acid residues are added to a fragment of a CCR11 amino acid sequence. The additional residues may be derived from another protein, or may include one or more residues that are not identifiable as being derived from a specific protein.

The invention also embraces CCR11 variants having additional amino acid residues which result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as part of glutathione-S-transferase (GST) fusion product provides the desired polypeptide having an additional glycine residue at position -1 after cleavage of the GST component from the desired polypeptide. Variants which result from expression in other vector systems are also contemplated.

Insertion variants may be fusion proteins wherein the amino and/or carboxy termini of the CCR11-polypeptide is fused to another polypeptide. Examples of other polypeptides are immunogenic polypeptides with long circulating half life

such as immunoglobulin constant regions, marker proteins (*e.g.*, fluorescent, chemiluminescence, enzymes, and the like), proteins or polypeptides that facilitate purification of the desired CCR11 polypeptide, and polypeptide sequences that promote formation of multimeric proteins (such as leucine zipper motifs that are useful in dimer formation/stability). Fusion proteins wherein a CCR11 polypeptide is conjugated to a hapten or other agent to improve, *i.e.*, enhance, immunogenicity, are also provided.

In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a CCR11 polypeptide are removed. Deletions can be effected at one or both termini of the CCR11 polypeptide, or with removal of one or more residues within the CCR11 amino acid sequence. Deletion variants, therefore, include all fragments of a CCR11 polypeptide. Disclosure of the complete CCR11 amino acid sequence necessarily makes available to the worker of ordinary skill in the art every possible fragment of the CCR11 polypeptide.

The invention also embraces polypeptide fragments of the sequence set out in SEQ ID NO: 2 wherein the fragments maintain biological, immunological, physical, and/or chemical properties of a CCR11 polypeptide. Fragments comprising at least 5, 10, 15, 20, 25, 30, 35, or 40 consecutive amino acids of SEQ ID NO: 2 are comprehended by the invention. Preferred polypeptide fragments display antigenic and/or other properties unique to the CCR11 family of polypeptides. Fragments of the invention having the desired properties can be prepared by any of the methods well known and routinely practiced in the art.

In still another aspect, the invention provides substitution variants of CCR11 polypeptides. Substitution variants include those polypeptides wherein one or more amino acid residues of a CCR11 polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are also non-conservative. Conservative substitutions for this purpose may be defined as set out in Table I.

Amino acids can be classified according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that

has similar properties. Exemplary conservative substitutions are set out in Table I, immediately below.

Table I
Conservative Substitutions

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe,
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

Substitution variant polypeptides include those wherein conservative substitutions have been introduced by modification of polynucleotides encoding polypeptides of the invention.

The invention also provides derivatives of CCR11 polypeptides. Derivatives according to the invention are CCR11 polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. The invention

specifically contemplates derivatization of CCR11 fragment polypeptides. Preferably, the modifications are covalent in nature, and include for example, chemical bonding with polymers, lipids, and other organic and inorganic moieties. Derivatives of the invention may be prepared to increase the circulating half-life of a CCR11 polypeptide, to improve targeting capacity for the polypeptide to desired cells, tissues, or organs, and/or to modulate (increase or decrease) CCR11.

The invention embraces derivative CCR11 polypeptides including one or more water soluble polymer attachments such as polyethylene glycol, polyoxyethylene glycol, polypropylene glycol or any of the many other polymers well known in the art, including, for example, monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol, as well as mixtures of these polymers. Particularly preferred are CCR11 products covalently modified with polyethylene glycol (PEG) subunits. Water soluble polymers may be bonded at specific positions, for example at the amino terminus of the CCR11 products, or randomly attached to one or more side chains of one or more amino acid residues in the polypeptide.

The invention further comprehends CCR11 polypeptides having combinations of insertions, deletions, substitutions, or derivatizations.

The following clone, p1 21679 was deposited with the American Type Culture Collection (ATCC) 10801 University Avenue, Manassas, Virginia, on March 2, 2000 under the terms of the Budapest Treaty (Accession No. PTA-1439). The clone represents a plasmid clone as described in the Examples set forth below.

Also comprehended by the present invention are antibodies [*e.g.*, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, bispecific antibodies, and complementary determining region (CDR)-grafted antibodies/proteins, including compounds which include CDR and/or antigen-binding sequences] and other binding proteins specific for CCR11 polypeptides of the invention as well as binding proteins that specifically bind an extracellular epitope or the amino terminal

extracellular domain of a CCR11 polypeptide. Preferred antibodies of the invention are human antibodies which are produced and identified according to methods described in WO93/11236, published June 20, 1993, which is incorporated herein by reference in its entirety. Antibody fragments, including Fab, Fab', $F(ab')_2$, and Fv, are also provided by the invention. The term "specific for" indicates that the variable regions of the antibodies of the invention recognize and bind CCR11 polypeptides exclusively (*i.e.*, are able to distinguish CCR11 polypeptides from the family of CC receptor polypeptides despite sequence identity, homology, or similarity found in the family of polypeptides), but may also interact with other proteins (for example, *S. aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable or CDR regions of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding specificity or exclusivity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow *et al.* (Eds), *Antibodies A Laboratory Manual*; Cold Spring Harbor Labbratory; Cold Spring Harbor , NY (1988), Chapter 6. Antibodies that recognize and bind fragments of the CCR11 polypeptides of the invention are also contemplated, provided that the antibodies are first and foremost specific or exclusive for, as defined above, CCR11 polypeptides. As with antibodies that are specific for full length CCR11 polypeptides, antibodies of the invention that recognize CCR11 fragments are those which can distinguish CCR11 polypeptides from the family of CC receptor polypeptides despite inherent sequence identity, homology, or similarity found in the family of proteins.

Antibodies of the invention can be produced using any method well known and routinely practiced in the art using any polypeptide or immunogenic fragment thereof of the invention. Immunogenic polypeptides can be isolated from natural sources, from recombinant host cells, or can be chemically synthesized. Polypeptides of the invention may be conjugated to a hapten such as keyhole limpet hemocyanin (KLH) in order to increase immunogenicity. Methods for synthesizing such peptides are known in the art, for example, as in R. P. Merrifield, *J. Amer. Chem. Soc.*, *85*: 2149-2154 (1963); J. L. Krstenansky, *et al.*, *FEBS Lett.*, *211*:10 (1987). Antibodies to a polypeptide of the invention can also be prepared through

immunization using a polynucleotide of the invention, as described in Fan *et al., Nat. Biotech.*, *17*:870-872 (1999). DNA encoding a polypeptide may be used to generate antibodies against the encoded polypetide following topical administration of naked plasmid DNA or following injection, and preferably intramuscular injection, of the DNA.

Non-human antibodies may be humanized by any methods known in the art. In one method, the non-human CDRs are inserted into a human antibody or consensus antibody framework sequence. Further changes can then be introduced into the antibody framework to modulate affinity or immunogenicity.

Antibodies of the invention further include plastic antibodies or molecularly imprinted polymers (MIPs) [Haupt and Mosbauch, *TIBTech*, *16*:468-475) (1998)]. Antibodies of this type are particularly useful in immunoaffinity separation, chromatography, solid phase extraction, immunoassays, for use as immunosensors, and for screening chemical or biological libraries. A typical method of preparation is described in Haupt and Mosbauch [*supra*]. Advantages of antibodies of this type are that no animal immunization is required, the antibodies are relatively inexpensive to produce, they are resistant to organic solvents, and they are reusable over a long period of time.

Antibodies of the invention can also include one or more labels that permit detection of the antibody, and in particular, detection of antibody binding. Labels can include, for example, radioactivity, fluorescence (or chemiluminescence), one of a high affinity binding pair (*e.g.*,biotin /avidin), enzymes, or combinations of one or more of these labels.

Antibodies of the invention are useful for, for example, therapeutic purposes (for modulating properties of CCR11), diagnostic purposes to detect or quantitate CCR11, as well as purification of CCR11. Kits comprising an antibody of the invention for any of the purposes described herein are also comprehended. In general, a kit of the invention includes a control antigen for which the antibody is immunospecific. Kits of the invention optionally include a container and/or a label.

The DNA and amino acid sequence information provided by the present invention also makes possible the systematic analysis of the structure and

function of CCR11. DNA and amino acid sequence information for CCR11 also permits identification of ligands and/or modulators with which a CCR11 polypeptide or polynucleotide will interact. Assays useful for identifying ligands and/or modulators are also provided. These assays may involve direct detection of CCR11 activity, for example, by monitoring binding of a labeled ligand to CCR11. In addition, these assays may be used to indirectly assess ligand interactions with CCR11. Identification of ligands and/or modulators of CCR11 polypeptides provides potential targets for therapeutic or prophylactic intervention in pathologies associated with CCR11.

Ligands can be identified or screened using isolated or recombinant CCR11 polypeptides or cells expressing such polypeptides. Ligands are manifestly useful in modulating (*i.e.*, blocking, inhibiting, or stimulating) CCR11, especially the signal transduction pathways wherein CCR11 participates directly or indirectly. Using binding assays, the inventors have confirmed that the chemokines ELC, SLC, and TECK are ligands for CCR11, as reported in Gosling *et al., J. Immunol., 164*: 2851-2856 (2000).

A number of assays useful for identifying chemokine ligands have already been described above. Such assays are useful with respect to the CCR11 receptor materials identified herein as an aspect of the invention. Likewise, methods described herein with specific reference to CCR11 have applicability to other chemoattractant receptors.

Direct detection of ligand binding to a chemokine receptor may be achieved using the following assay. Test compounds (*i.e.*, putative ligands) are detectably labeled (*e.g.*, radioiodinated). The detectably labeled test compounds are then contacted with membrane preparations containing a CCR11 polypeptide of the invention. Preferably, the membranes are prepared from host cells expressing CCR11 of the invention. Following an incubation period to facilitate contact between the membrane-embedded polypeptide and the detectably labeled test compounds, the membrane material is collected on filters using vacuum filtration. The detectable label associated with the filters is then quantitated. For example, radiolabels are quantitated using liquid scintillation spectrophotometry. Using this technique, ligands

binding to CCR11 are identified. To confirm the identification of a ligand, a detectably labeled test compound is exposed to a membrane preparation displaying CCR11 in the presence of increasing quantities of the test compound in an unlabeled state. A progressive reduction in the level of filter-associated label as one adds increasing quantities of unlabeled test compound confirms the identification of that test compound as a ligand.

Modulators of CCR11 may be identified using a similar assay. The membrane preparation displaying CCR11 is exposed to a constant and known quantity of a labeled functional ligand. The membrane-bound CCR11 is also exposed to an increasing quantity of a test compound suspected of modulating the activity of CCR11. If the levels of filter-associated label correlate with the quantity of test compound, that compound is a CCR11 modulator . If the level of filter-associated label increases with increasing quantities of the test compound, a stimulatory modulator has been identified. In contrast, if the level of filter-associated label varies inversely with the quantity of test compound, an inhibitor of CCR11 has been identified. Confirmation of the identification of a modulator can be achieved by competing a differently labeled form of the modulator with varying quantities of an unlabeled form of the modulator using the assay described above for confirming ligand identities.

Indirect assays for identifying ligands and modulators of CCR11 involve measurements of the concentration or level of activity of any of the components found in the CCR11 signal transduction pathway. Chemokine receptor activation is often associated with an intracellular Ca^{++} flux. Cells expressing CCR11 may be loaded with a Ca^{++}-sensitive dye. Upon activation of CCR11, a Ca^{++} flux would be rendered spectrophotometrically detectable by the dye. Alternatively, Ca^{++} flux could be measured microscopically. Parallel assays, using either technique, may be performed in the presence or absence of putative ligands.

Alternatively, the association of CCR11 with G proteins affords the opportunity of assessing CCR11 activity by monitoring G protein activities. A characteristic activity of G proteins, GTP hydrolysis, may be monitored using, for example, ^{32}P-labeled GTP. G proteins also affect a variety of other molecules

downstream in their signal transduction pathways. For example, G protein effector molecules include adenylyl cyclase, phospholipase C, ion channels and phosphodiesterases. Assays focused on any of these effectors may be used to monitor CCR11 activity induced by ligand binding in a host cell that is both expressing CCR11 and contacted with an appropriate ligand. Those skilled in the art will recognize that these assays are useful for identifying and monitoring the purification of modulators of receptor activity.

Another indirect assay of the invention is a chemotaxis-based assay that may be used to rapidly identify or select cells expressing a functional CCR11 polypeptide that is capable of mediating such chemotaxis. Alternatively, the chemotaxis-based method is used to identify CCR11 ligands based upon the ability of a putative ligand, whether natural or synthetic, to elicit chemotaxis of CCR11-expressing cells.

Chemotaxis-based assays may be employed in a number of mutually complementary ways to provide information about CCR11 polypeptides, cells that express the receptor, and ligands of the receptor. In one approach, CCR11, which has now been characterized as a chemoattractant receptor, may be demonstrated to have additional ligands or to have no other known ligands. In another approach, a cell type of interest may be tested to determine whether it expresses CCR11. In still another approach, the method may be used to define a characteristic chemoattractant profile for a cell expressing CCR11, by screening against a number of chemoattractants. In yet another embodiment, the method may be used to identify modulators, *e.g.*, competitive or non-competitive agonists or antagonists, of CCR11. The modulators identified thereby may be expected to have useful properties and to lead to therapeutic candidate compounds. Moreover, in another embodiment, a putative chemoattractant compound may be screened against a plurality of receptors including CCR11 (as they are expressed on cells) to determine whether the compound has chemoattractant properties. Other advantages will present themselves in particular contexts.

Additional embodiments, features, and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including

the Drawing and the Detailed Description, and all such features are intended as aspects of the invention.

Likewise, features of the invention described herein can be re-combined into additional embodiments that also are intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the invention should be viewed as such; variations of the invention lacking limitations which have not been described herein as critical are intended as aspects of the invention.

With respect to aspects of the invention that have been described as a set or genus, every individual member of the set or genus is intended, individually, as an aspect of the invention.

In addition to the foregoing, the invention includes, as an additional aspect, all embodiments of the invention narrower in scope in any way than the variations specifically mentioned above. Although the applicant(s) invented the full scope of the claims appended hereto, the claims appended hereto are not intended to encompass within their scope the prior art work of others. Therefore, in the event that statutory prior art within the scope of a claim is brought to the attention of the applicants by a Patent Office or other entity or individual, the applicant(s) reserve the right to exercise amendment rights under applicable patent laws to redefine the subject matter of such a claim to specifically exclude such statutory prior art or obvious variations of statutory prior art from the scope of such a claim. Variations of the invention defined by such amended claims also are intended as aspects of the invention.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a dendrogram analysis which illustrates the similarity of the deduced amino acid sequence of CCR11 with other CC chemokine receptors. Percentages of identity with CCR11 are shown at the right of the figure.

Figure 2 is a graph depicting results of a ligand binding assay using human CCR11. The results show displacement of ^{125}I-ELC binding to HEK293 cells

expressing CCR11 when the cells are incubated with unlabeled chemokines. Cells were incubated with ^{125}I-ELC in the presence of the indicated concentrations of unlabeled chemokines for one hour at room temperature. Cells were washed three times, then the amount of ^{125}I-ELC bound was determined. IC_{50}'s for this experiment were ELC, 4.75 nM; SLC, 68.7 nM, TECK, 38.1 nM; MCP-4, undetermined.

DETAILED DESCRIPTION OF THE INENTION

A number of assays useful for identifying chemokine ligands have already been described above. Such assays are useful with respect to the CCR11 receptor materials identified herein as an aspect of the invention. Likewise, methods described herein with specific reference to CCR11 have applicability to other chemoattractant receptors.

We have developed a method that allows for the rapid selection of cells expressing functional receptors for chemoattractants, such as the CCR11 receptor. In particular, the presently described method permits the artisan to identify extraordinarily rare subpopulations of cells expressing functional receptors. Compared to methods commonly employed in the art, assay sensitivity can be increased several orders of magnitude. For example, using the materials and methods of the invention permits selection of 1 in 1,000 to 1 in 1,000,000 cells. This translates to an increase in assay sensitivity of order 10^3 to 10^6. Given that it is intrinsically difficult to reliably generate functional chemokine (or other chemoattractant) receptors by transfection of recombinant orphan receptors into cells, such sensitivity enhancement may be crucial to detecting a ligand interaction with a receptor. As will be made evident below, the method is capable of demonstrating ligand/receptor relationships in cases where conventional assays have been unable to detect any functional interaction.

Chemotaxis or chemotactic migration may be broadly defined as stimulated motility or orientation by cells that is directional with respect to a chemical stimulus. Alternatively, chemotaxis may be defined as the directional response of biological cells or organisms to chemical concentration gradients. Chemotaxis is differentiated from chemokinesis, in which the motion or orientation of the cells is

non-directional or random. Chemotaxis may or may not be evident by examining a single cell, as cellular motility is not typically uniformly directional. That is, a cell may be exhibiting a response that is properly understood as chemotaxis, yet appear to be moving randomly or in no particular direction. Chemotaxis is, however, demonstrable as a statistical phenomenon when examining a cell population. Chemokinesis can be distinguished from chemotaxis, *e.g.*, by a checkerboard analysis, as described by Zigmond and Hirsch, *J Exp Med, 137*: 387-410 (1973).

The invention enables the skilled artisan to perform numerous types of cell- based assays for detection or identification of functional receptor/ligand pairs. The method can be easily adapted to the specific functional or biological features of the receptors and/or test compounds of interest. Applications include, for example, rapid identification or isolation of cells expressing a functional receptor that is capable of mediating stimulus of chemotaxis, and the identification of ligands for orphan receptors, *i.e.*, receptors for which functions and/or ligands have not been identified. Identification of receptors as chemoattractant receptors and, likewise, identification of test compounds as chemoattractant ligands, is based on its ability to elicit chemotaxis of the cells.

Thus, the invention may be directed toward the characterization of a receptor of interest. One important use of the invention is to characterize orphan receptors whose function has not previously been characterized. The invention may also be used to further characterize receptors whose function in cells in which they are natively expressed is already partially characterized. For example, when a receptor is known to mediate chemotaxis in response to specific ligands, the invention may be used to identify additional chemoattractant ligands capable of eliciting a similar response.

Alternatively, the invention may be directed to characterization of test compounds suspected of having chemoattractant function. Thus, the invention may be used to characterize test compounds that have not previously been recognized as chemoattractants. The invention may also be used to further characterize test compounds that have previously been shown to act as chemoattractants through interaction with other receptors. For example, a known chemoattractant compound

may be tested against one receptor or a panel comprising a plurality of receptors to determine whether the test compound possesses additional chemoattractant capacity.

Indeed, it is one of the virtues of the present invention that, by using methods described herein, receptors and test compounds that are each uncharacterized as to their properties as chemoattractant receptors or as chemoattractants, respectively, may be identified as sharing a functional receptor/ligand relationship. Thus, a panel of orphan receptors and a panel of test compounds may be tested in an array format to reveal "hits," *i.e.*, those receptor/ligand pairs in which a chemotactic response is observed. Similarly, as demonstrated herein, partially characterized receptors and ligands may be employed to screen for novel functional relationships.

The invention may be employed in a number of mutually complementary ways. In one approach, the method may be used to characterize an orphan receptor as a chemoattractant receptor. In another approach, a previously characterized chemoattractant receptor may be demonstrated to have additional functional ligands or to have no other known ligands. In another approach, a cell type of interest may be tested to determine whether it expresses a chemoattractant receptor. In still another approach, the method may be used to define a characteristic chemoattractant profile for a cell type or a receptor of interest, by screening against a number of chemoattractants. In yet another embodiment, the method may be used to identify modulators, *e.g.*, competitive or non-competitive agonists or antagonists, of a chemoattractant receptor of interest. The modulators identified thereby may be expected to have useful properties and to lead to therapeutic candidate compounds. Moreover, in another embodiment, a putative chemoattractant compound may be screened against a plurality of receptors (as they are expressed on cells) to determine whether the compound has chemoattractant properties. Other advantages will present themselves in particular contexts. The skilled artisan, therefore, will recognize that the invention may be implemented in a variety of methodological embodiments as desired.

The invention provides methods of screening receptor-expressing cells using soluble chemoattractant ligands. In this approach, an initial gradient exists across the barrier membrane, which is diminished as the soluble ligand diffuses or

equilibrates across the membrane. It may be useful in some cases to define a gradient that is maintained more or less stable, *i.e.*, equilibrates not at all or at a reduced rate. Any method of reducing equilibration of the gradient may be used. For example, the ligand may be provided a matrix from which it is slowly released, to provide a continuous concentration differential across the membrane. Suitable matrix materials include, for example, water-soluble polymers and hydratable polymers. Any biocompatible or physiologically compatible matrix material can be used, such as those materials used in medicaments reliant on sustained release formulations. As chemokines and other chemoattractants are typically highly basic, the matrix may advantageously include acidic moieties to promote association with the matrix. Exemplary matrix materials include, for example, sulfated proteins and proteoglycans, as such materials often underlie chemoattractant gradients *in vivo*.

The method may be employed with or without further treatment of the cells to modulate their responsiveness. This type of parallel treatment with another factor may reveal additional information about the functional status of the receptor of interest. For example, the cells may be pre-challenged with a compound that induces some change in activation state of the cells such that their response to the chemoattractant(s) is depressed or augmented.

The chemotaxis assay of the invention is carried out by detecting migration of cells through a cell-permeable barrier, wherein migration occurs in the direction from the first surface of the barrier through to the opposite, second surface, wherein a known or suspected chemoattractant is situated below the second surface at increased levels relative to the levels present above the first surface such that the direction of migration is toward the chemoattractant.

Thus, chemotactic activity is measured by first establishing a concentration gradient in the chemotaxis chamber. The chamber is incubated for a predetermined time, and then the filter is removed from the apparatus. The cells that have migrated through the filter into the lower chamber (or into the filter to a certain depth) are then counted. A comparison is then made between the activity of the cells in a concentration gradient of the chemotactic factor being tested, and the activity of the cells in the absence of the concentration gradient.

Although various designs are possible, conventional chemotaxis chambers typically comprise two compartments or chambers arranged in a stacked configuration. The upper and lower chambers are separated by a cell-permeable barrier that defines a bottom surface of the upper chamber and a top surface of the lower chamber. Typically, one or both of the chambers are open to air. The apparatus is designed so that, under appropriate conditions, cells that are initially placed in the upper chamber can migrate or "crawl" through the barrier into the lower chamber whereupon they are counted to measure the amount of chemotaxis that occurs. The cell permeable barrier should be situated to be in contact with both the fluid containing the cellular composition in the upper chamber as well as the fluid containing the chemoattractant/test molecule in the lower chamber.

Microchemotaxis chambers and some of their applications are well known in the art, and are described in numerous publications. See, for example, Falk *et al.*, *J Immunol Methods, 33*: 239-47 (1980); Harvath *et al.*, *J Immunol Methods, 37*: 39-45 (1980); Richards *et al.*, *Immunol Commun, 13(1)*: 49-62 (1984); Falk *et al.*, *Infect Immun, 36*: 450-54 (1982); and Harvath *et al.*, *Infect Immun, 36(2)*: 443-49 (1982). See also, P. Wilkinson, *Methods Enzymol, 162*: 38-50 (1988).

The chambers of the chemotaxis apparatus can be formed from various solid phase materials, including but not limited to glass, quartz, and plastic (*e.g.*, polystyrene, Lucite®, polypropylene, polycarbonate, and the like), with plastic being preferred. The apparatus may be formed as a unit, or assembled to form a unit. Preferably, the chambers are separate parts, which may fit together or interlock, and are preferably detachable from each other. The chambers may also be detachable from the filter, to facilitate setup of the assay and counting of cells after chemotaxis has occurred. However, the filter is typically affixed to and defines the bottom of the upper chamber. Optionally, each chamber has one or more ports, inlet means, or other openings for injection of solutions. Exemplary commercially available chemotaxis apparatus includes the Transwell® tissue culture inserts available from Costar (Cambridge MA). Snapwell® diffusion chambers (Costar) are also amenable to use according to the invention. Chemotaxis plates comprising an array of discrete wells are valuable for high throughput screening procedures in which a plurality of

receptors and/or a plurality of test compounds are simultaneously tested. As noted, the barrier should be permeable to the cells, but should inhibit distribution of the cells into the target volume by means other than their own migration, *e.g.*, gravity. Thus, the barrier may contain pores of a controlled size through which cells can actively migrate.

In some embodiments of the present invention, the cell permeable barrier is a microporous filter or membrane, *e.g.*, a capillary pore membrane filter. The pores in the microporous barrier may be between about 0.1 μm and about 20 μm in average diameter, preferably between about 2 μm and about 15 μm, depending on the type of cell that is being used in the assay, and the nature of the assay. For example, a 0.1 μm or 0.2 μm pore size can be used to allow the pseudopods of certain cells (*e.g.*, cancer cells) to penetrate the barrier in response to a chemotactic factor, but preclude the cell bodies from getting through the barrier. The differential response is then measured by determining how much the pseudopods protrude in stimulated as opposed to unstimulated wells. However, the assays according to the invention are preferably read by counting migrated cells. Accordingly, the pores of the barrier must be large enough to permit those cells to migrate through the pores, typically averaging in the range of from about 2 μm to about 10 μm; more often from about 3 μm to about 8 μm.

The thickness of the microporous barrier can be selected based on the desired application. However, the barrier is typically from about 5 μm to about 30 μm thick.

Membrane or filter barriers may be manufactured of any biocompatible material, including polymeric materials such as polytetrafluoroethylene (PTFE), polyester, nylon, polycarbonate, and the like. Polystyrene may be preferred for easy disposability. More durable materials such as polycarbonate may be preferred for reusable apparatus.

In alternative embodiments, a water-soluble or hydratable gel material may be provided that is permissive to cell migration. In this case, the physical barrier limiting non-specific movement of the cells is provided by inherent physical limitations of the material; the material is selected such that the porosity requires cells

to migrate into and/or through the material. Indeed, a gel material may be provided that promotes cell migration, *e.g.*, Matrigel®, by providing a matrix that is adapted to be compatible with the physical processes employed by cells in the migration process. The method can employ combinations of these barriers. Thus, the apparatus in which the chemotaxis is to occur may contain a membrane barrier together with a gel barrier.

The barrier may comprise a coextensive layer, *e.g.*, a monolayer, of cells according to techniques known in the art (such as a layer of endothelial cells as described in U.S. Patent No. 5,514,555 to Springer *et al.*), to more closely mimic the milieu of cells whose typical in situ response to chemoattractants may include traversing a cellular barrier. Epithelial layers may be employed as the principal barrier without underlying microporous membrane, provided they have adequate structural support or integrity. An exemplary epithelial cell type useful in establishing cell layers is the A549 epithelial cell line. To illustrate, a membrane of a higher porosity may be used as a support for the cells while itself interposing no substantial barrier to cell movement.

The barrier may comprise a coextensive coating of a composition that is conducive to cellular transmigration, *e.g.*, collagen, fibronectin, fibrinogen, laminin, gelatin, *etc.* Preferably, the coating is provided on the surface of the barrier that contacts the cells to promote adherence and transmigration, however, such coatings may be provided on either or both surfaces. It is also preferred that the physical structure of the coating does not substantially interfere with the permeability of the barrier.

The cells are provided in a medium, preferably an aqueous medium, such as the medium in which they are grown (*e.g.*, a balanced salt and nutrient solution) or a buffered physiologically compatible medium such as phosphate buffered saline (PBS). The assay medium preferably comprises cell culture medium, for example, RPMI 1640 (*e.g.*, from Gibco) (or L15) plus M199 (*e.g.*, Gibco) (preferably in a 1:1 ratio). It is important to add some protein such as human serum albumin (HSA), bovine serum albumin (BSA), or fetal bovine serum (FBS) to the fluid in both the upper and lower chambers, to a final concentration in the range of 0.25-1%; the same protein need not be present in both chambers. Dilutions of test

compounds are preferably carried out in fluid identical to that present in the chamber to which the test compound is to be added. Preferably, the assay medium is at least substantially free of components that induce migration of the cells.

The cells useful according to the method of the invention comprise cells that express a cell surface protein that is capable of mediating a cellular response to a chemoattractant, *i.e.*, a functional chemoattractant receptor, such as CCR11 descibed herein. The functional receptor may be natively expressed by (*i.e.*, endogenous to) the responding cells. Preferably, the receptor is exogenous to the cells, *i.e.*, it is not natively expressed by the cells or is not encoded in the genome of the species from which the cells are derived. In this latter case, the receptor is recombinantly expressed following transfection into the cells by conventional means. The cells may natively express a functional receptor that is responsive to one or more other chemoattractant and yet still be useful as host cells for expression of a receptor of interest, provided that the two receptors exhibit different responsiveness profiles. Thus, in one embodiment a cell that is normally responsive to a first chemoattractant may be modified to express a receptor that binds the same chemoattractant. In such cases, a modest chemotactic capacity by the unmodified cells may be measurably supplemented by the uncharacterized receptor, even though the signal-to-noise ratio may not be as favorable as might be the case when using a cell type that is normally completely unresponsive to the chemokine. This scenario is also less desirable inasmuch as cloning of cells expressing one or both of two functionally undifferentiated receptors cannot guarantee that any given clone will express the receptor of interest. This limitation can be overcome by complementary characterization assays of other types or by analysis of the comparative responsiveness of the cell of interest to that of unmodified cells, which will have a characteristic profile of responsiveness.

The cells useful according to chemotaxis assays of the invention must be capable of undertaking directed or chemotactic migration when appropriately stimulated to do so. Preferably, the cells exhibit no or little spontaneous or non-specific migration (chemokinetic) activity. However, it is desirable that the cells have strong migration capacity so that a chemotactic signal can be readily detected

even if a particular ligand is not a primary or potent chemoattractant for the receptor of interest. Cells susceptible to chemokinetic stimulation may be used. Mammalian cells are preferred. Exemplary cells useful according to the invention include cells of hematopoietic origin, *e.g.*, lymphocytes such as the HUT78 T cell line, monocytes such as the U937 monocytic cell line, macrophages, dendritic cells, polymorphonuclear leukocytes (PMNs) such as neutrophils, eosinophils, and basophils), and pre-B cells such as murine L1.2 cells.

The host cell population may be heterogeneous, *i.e.*, comprising cells of different phenotypes. Preferably, the host cell population consists essentially of cells of a single phenotype of interest, and more preferably, consists of cells of uniform phenotype. Typically, the host cell population will be composed predominantly of cells of a desirable phenotype, while containing a minority of cells having phenotypes differing along one or more parameters from the core population. Substantially uniform cell populations are preferred. Nonetheless, transfection of a host cell line with an expression vector comprising DNA encoding a receptor of interest may yield cells whose phenotype shifts over time. Moreover, expression of chemoattractant receptors such as GPCRs often yields a modified cell population that is heterogeneous with respect to the level of expression of the receptor as well as functional integration of the receptor into the cellular physiology. Thus, many transfected cells may express a receptor that is not functional, and purification methods such as antibody-based typically cannot discriminate between functional and non-functional receptors. Consequently, the modified population may not exhibit sufficient responsiveness in assays of ligands for the receptors. Historically, this has been a problem in the field of chemokine receptor characterization, as expressed exogenous receptors may lose functionality over time.

To assess chemotaxis, the lower chamber of the chemotaxis apparatus contains a solution of a test compound in the assay medium. A "test compound" as used herein refers to a molecule being tested for the desired activity in promoting or inhibiting chemotaxis, as the case may be. Preferred test compounds include compounds known to have chemoattractant properties, such as chemokines, chemotactic lipids (*e.g.*, LTB_4), chemoattractant peptides other than chemokines, and

the like. Compounds of similar constitution, but not known to be chemoattractants, may be screened to determine whether they have such a property. Alternatively, compounds of different chemical structure may be screened to determine whether they function as chemoattractants, *i.e.*, functional analogs. For example, chemical libraries, including, *e.g.*, peptides, peptidomimetics, small organic molecules, natural products, etc., and comprising tens to hundreds of thousands of compounds, may be screened in accordance with the invention to identify compounds having chemoattractant activity (e.g., as ligands for CCR11).

A composition comprising the test compound in solution may be added to the lower chamber simultaneously with, or shortly before or after, addition of the cellular composition to the upper chamber. Except for added cells or test compounds as stated above, the fluids in the upper and lower chambers are preferably the same or substantially similar. In a kit form, the test compound may be provided in a pre-determined amount of the test compound(s) deposited in dried form in the bottom of the lower chamber to be dissolved in a specified amount of the assay medium when the assay is conducted. The test compound or chemoattractant compound can be used in various dilutions to derive a dose-response curve.

The controls are generally of two kinds: negative controls and chemokinetic controls. Negative controls will include assay medium alone in the lower chambers. Alternatively, chemokinetic controls contain the same concentration of test compound in the top and lower chambers; *i.e.*, above and below the barrier. Chemokinetic controls differ from the chemotaxis test sites because there is no gradient in the concentration of the test compound. Chemokinetic controls allow the user to distinguish heightened random activity of the cells, due to contact with the test compound, from directional response in a concentration gradient of the test compound.

The method of the invention may be employ a chemokinetic compound that increases general motility of cells being assayed. To illustrate, in the case of leukocytes, histamine may be employed to non-specifically increase cellular motility. However, this approach will typically be less preferred as an overall increase in the

motility of the cells can increase the background (noise) levels of migration, thereby lowering the relative sensitivity of the method.

After placement of the cellular composition and chemoattractant composition in the upper and lower chambers, respectively, the apparatus is incubated to allow any chemotaxis by the cells to take place. Incubation is carried out for about 3-6 hours, and is preferably done for about 4 hours. The temperature of incubation is typically from about 25°C to about 40°C, depending on cell type, and is preferably about 37°C for many mammalian cell types. Incubation may be carried out in room air or in a controlled atmosphere, as some cell types and some growth media require specialized gas mixtures. For example, growth of some cells or use of some media may require 5% CO_2.

Following incubation, chemotaxis is detected or measured by determining data relative to a control or background level of migration into the lower chamber measured in which no chemoattractant or test molecule is placed in the lower chamber. An increased number of cells in the lower chamber relative to the background level indicates chemotaxis (or chemokinesis) has occurred. Any suitable detection or quantitation method may be used. For example, if the cells placed in the upper chamber are labeled, the label can be detected and/or measured. Cells in the lower chamber can be counted by fluorescent microscopy or quantitated in a fluorescence concentration analyzer. Alternatively, the cells can be retrieved from the lower chamber and counted directly under the microscope, *e.g.*, using a hemacytometer. Another useful method is to collect the fluid in the lower chamber along with the cells, and then count the cells by flow cytometry. When multiwell or microtiter filter plates are employed, cells in the bottom wells can be collected and counted once the top plate is removed. Many methods are available to the skilled artisan. Data can be expressed by any convenient method, *e.g.*, as absolute number of cells or as percentage of input cells migrating into the lower chamber.

In a specific embodiment in which migrating cells are detected or measured by detecting and/or quantitating a label appearing in the lower chamber of the apparatus, the cells are labeled before placing them in the upper chamber. This can be accomplished by any of various methods known in the art, *e.g.*, by fluorescent

labeling of the cells, enzymatic labeling (*e.g.,* via an enzyme-tagged antibody to a cell surface marker), etc. In a preferred aspect, cells are fluorescently labeled with fluorescein or a derivative thereof such as 2',7'-bis-(2-carboxyethyl)-5(or 6)-carboxyfluorescein (BCECF) or calcein (Molecular Probes, Eugene OR).

In U.S. Patent No. 5,601,997 to Tchao, there is described a method for assaying chemotaxis in which the cells are pre-labeled with a fluorescent dye. The cells that have migrated to the lower chamber are then counted using a fluorescent plate reader, *e.g.*, Cytofluor® 2300 (Millipore Corp., Marlborough MA). Use of a radiation opaque membrane eliminates false positive counts, as cells above the membrane are shielded from the excitation light and only the cells that have migrated through the membrane absorb the light and emit. Alternatively, when the assay apparatus permits, simple removal of the upper chamber avoids confounding signal from the unmigrated cells.

The method of the invention can be employed to potentiate assays of biological molecules implicated in chemoattractant receptor signaling, including such biological molecules as kinases, phosphatases, arrestins, and regulator of G-protein signaling (RGS). The invention enables large increases in the sensitivity in these assays, which depend on a sequence of events within the cell to produce a readable signal.

Several exemplary embodiments are described below. These embodiments are provided for purposes of illustrating the invention and should not be interpreted as limiting the scope of the invention.

In one embodiment, the invention provides a method of culturing cells expressing a cell surface receptor that mediates chemotaxis. In this approach, the method may comprise steps of culturing the cells in a medium that supports growth and proliferation of the cells; subjecting the cells to a chemoattractant stimulus to separate cells that exhibit a chemotactic response from cells that exhibit no significant chemotactic response; and selecting cells that exhibit a chemotactic response for continued culturing in a medium that supports growth and proliferation of the cells. This method can be used to provide a culture of cells enriched in cells capable of chemotaxis in response to the chemoattractant stimulus. The method may further

comprise exposing the enriched cells to subsequent steps of subjecting the cells to the chemoattractant stimulus and selecting the cells that exhibit the desired chemotactic response.

In another embodiment, the invention involves screening cells that express an exogenous cell surface receptor to determine whether the receptor mediates chemotaxis by the cells. In this case, the method comprises the steps of: providing cells that have been modified to express the receptor; contacting the receptor-expressing cells with a test compound; and isolating cells that exhibit chemotactic migration in response to the test compound to provide a cell population expressing the receptor as a functional chemoattractant receptor that binds the test compound, whereby a chemotactic function is defined for the receptor, and whereby a chemoattractant ligand for the receptor is identified.

In another approach, the invention provides a method of identifying cells expressing a receptor that mediates chemotaxis. Thus, the method comprises the steps of providing a cell reservoir having a bottom comprising a cell permeable membrane having upper and lower surfaces, wherein the cell reservoir contains receptor expressing cells in a medium that is substantially free of agents that induce chemotaxis mediated by the receptor, and wherein the medium is in contact with the upper surface of the cell permeable membrane; providing a ligand reservoir containing a test medium comprising a test compound, wherein the test medium contacts the lower surface of the cell permeable membrane; incubating the cells for a time sufficient to allow chemotaxis of cells across the cell permeable membrane in response to the test compound; and collecting cells that migrate across the cell permeable membrane into the ligand reservoir. Using this method, one may obtain a cell population enriched for cells that exhibit chemotactic migration in response to the test compound.

In the embodiments described herein, the method may further comprise cloning a cell that has been selected or isolated based on its chemotactic activity and culturing the cloned cell in a medium that supports growth and proliferation of the cell. In this way, the artisan can establish a substantially homogeneous cell population consisting essentially of cells that migrate in response to the test compound.

Thus, the invention provides a method of providing a clonal population of cells expressing a chemoattractant receptor. For example, the method can comprise providing a cell population comprising cells that express a chemoattractant receptor of interest; contacting the cell population with a chemotaxis-inducing amount of a test compound that is a functional ligand of the receptor, under conditions that permit chemotaxis by cells in response to stimulus by the test compound; collecting a responsive cell subset comprising cells that exhibit chemotaxis in the presence of the test compound; and culturing an isolated cell from the responsive cell subset in a medium that supports growth and proliferation of the isolated cell. In this way, a clonal cell population can be established in culture.

For example, the invention provides a method of providing a clonal population of cells expressing CCR11. For example, the method can comprise providing a cell population comprising cells that express CCR11; contacting the cell population with a chemotaxis-inducing amount of a test compound that is a functional ligand of CCR11, under conditions that permit chemotaxis by cells in response to stimulus by the test compound; collecting a responsive cell subset comprising cells that exhibit chemotaxis in the presence of the test compound; and culturing an isolated cell from the responsive cell subset in a medium that supports growth and proliferation of the isolated cell. In this way, a clonal cell population that expresses functional CCR11 can be established in culture. It may be noted that the invention also enables the artisan to develop a substantially homogeneous population of cells expressing an exogenous chemoattractant receptor, wherein the proportion of cells in the population that express the receptor in a functional state is higher than in a population of cells directly obtainable by transfection of cells through an expression vector containing DNA encoding the receptor.

Accordingly, the invention provides a cell or cells expressing a functional exogenous chemoattractant receptor, isolable from cells transfected by an expression vector containing DNA encoding the receptor through the described method. Furthermore, the invention enables the artisan to develop a substantially homogeneous population of cells expressing an exogenous chemoattractant receptor, wherein the proportion of cells in the population that express the receptor in a

functional state is higher than in a population of cells directly obtainable by transfection of cells through an expression vector containing DNA encoding the receptor.

Other implementations of the invention are envisioned, and may be further adapted by the skilled artisan. To illustrate, the method of the invention may comprise screening cells expressing an orphan receptor whose chemoattractant responsiveness is uncharacterized. Thus, the method may comprise steps of providing a cell population comprising cells that have been modified to express an orphan receptor of interest; contacting the cell population with a plurality of test compounds that have chemoattractant activity; and measuring chemotaxis by the orphan receptor-expressing cells in the presence and absence of the plurality of test compounds. In this case, chemotaxis by the orphan receptor-expressing cells in the presence of one or more of the plurality of test compounds indicates that the orphan receptor is a chemoattractant receptor.

Alternatively, the method may be used to screen cells that express a receptor that is known to mediate chemotaxis. Here, the method may comprise steps of providing a cell population comprising cells expressing a known chemoattractant receptor; contacting the cell population with a plurality of test compounds whose chemoattractant activities are uncharacterized with respect to the chemoattractant receptor; and measuring chemotaxis by the receptor-expressing cells in the presence and absence of the plurality of test compounds. In this way, chemotaxis by the receptor-expressing cells in the presence of any of the plurality of test compounds indicates that that test compound is an agonistic ligand for the chemoattractant receptor.

Numerous implementations of the invention are envisioned, and may be further adapted by the skilled artisan. To illustrate, the method may be used to screen cells that express a receptor that is known to mediate chemotaxis. Here, the method may comprise steps of providing a cell population comprising cells expressing CCR11; contacting the cell population with a plurality of test compounds whose chemoattractant activities are uncharacterized with respect to CCR11; and measuring chemotaxis by the CCR11-expressing cells in the presence and absence of the test

compounds. In this way, chemotaxis by the CCR11-expressing cells in the presence of a test compound indicates that that compound is an agonistic ligand for CCR11. A panel of cells and a panel of test compounds may be tested in an array format to reveal "hits," i.e., those receptor/ligand pairs in which a chemotactic response is observed.

In addition, the method may involve determining the relative potency of a panel of chemoattractants. According to this approach, the method may comprise steps of contacting cells expressing a functional chemoattractant receptor with each of a plurality of chemoattractants; and counting the cells migrating in response to each of the plurality of chemoattractants. Using this approach, the number of cells migrating for each of the plurality of chemoattractants correlates directly with the relative potency of the chemoattractants in inducing migration of the receptor-expressing cells.

For example, the method may involve determining the relative potency of a panel of test compounds as functional chemoattractant ligands of CCR11. According to this approach, the method may comprise steps of contacting cells expressing CCR11 with each of a plurality of test compounds; and counting the cells migrating in response to each of the test compounds. Using this approach, the number of cells migrating for each of the test compounds correlates directly with the relative potency of the compounds in inducing migration of the CCR11-expressing cells.

In another approach, a method of screening a test compound for antagonism of a chemoattractant receptor may comprise steps of contacting cells expressing a functional chemoattractant receptor with a chemoattractant agonist known to induce migration of the receptor-expressing cells; contacting the receptor-expressing cells with a test compound; and counting the cells migrating in the presence and absence of the test compound. In this case, a reduction of the number of cells migrating in the presence of the test compound correlates with antagonism of migration of the receptor-expressing cells by the test compound.

For example, a method of screening a test compound for antagonism of CCR11 function may comprise steps of contacting cells expressing functional CCR11 with a chemoattractant agonist known to induce migration of the CCR11-expressing cells; contacting the receptor-expressing cells with a test compound; and counting the

cells migrating in the presence and absence of the test compound. In this case, a reduction of the number of cells migrating in the presence of the test compound correlates with antagonism of migration of the CCR11-expressing cells by the test compound.

Agents that modulate (*i.e.*, stimulate, inhibit, or block) CCR11 may be identified by incubating a putative modulator with a CCR11 polypeptide and determining the effect of the putative modulator on CCR11. The selectivity, or specificity, of a compound that modulates CCR11 can be evaluated by comparing its effects on CCR11 to its effect on other chemokine receptors.

Selective modulators may include, for example, antibodies and other proteins or peptides which specifically bind to a CCR11 polypeptide and other non-peptide compounds (*e.g.*, isolated or synthetic organic and inorganic molecules) which specifically react with a CCR11 polypeptide. Preferably, modulators of the invention will bind specifically or exclusively to a CCR11 polypeptide, however, modulators that bind a CCR11 polypeptide with higher affinity or avidity compared to other compounds are also contemplated. Mutant CCR11 polypeptides which affect wild-type CCR11 polypeptides or its cellular localization are also contemplated by the invention. Presently preferred targets for the development of selective modulators include, for example: (1) regions of a CCR11 polypeptide which contact other proteins, (2) regions that localize a CCR11 polypeptide within a cell, (3) regions of a CCR11 polypeptide which bind ligand, (4) allosteric regulatory binding sites of a CCR11 polypeptide, (5) phosphorlyation site(s) of a CCR11 polypeptide as well as other regions of the protein wherein covalent modification regulates biological activity, and (6) regions of a CCR11 polypeptide which are involved in membrane conformation. Still other selective modulators include those that recognize specific CCR11-encoding and regulatory sequences. Modulators of CCR11 activity may be therapeutically useful in treatment of diseases and physiological conditions in which CCR11 is known or suspected to be involved.

Methods of the invention to identify modulators include variations on any of the methods described above to identify binding partner compounds, the variations including techniques wherein a binding partner compound has been

identified and the binding assay is carried out in the presence and absence of a candidate modulator. *In vitro* methods of the invention are particularly amenable to high throughput assays as described below.

The invention also comprehends high throughput screening (HTS) assays to identify compounds that interact with or inhibit a CCR11 polypeptide. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based HTS systems are contemplated, including melanophore assays to investigate receptor-ligand interaction, yeast-based assay systems, and mammalian cell expression systems Jayawickreme and Kost, *Curr. Opin. Biotechnol.*, *8*:629-634 (1997). Automated (robotic) and miniaturized HTS assays are also embraced Houston and Banks, *Curr. Opin. Biotechnol. 8*:734-740 (1997). HTS assays are designed to identify "hits" or "lead compounds" having the desired property, from which modifications can be designed to improve the desired property. Chemical modification of the "hit" or "lead compound" is often based on an identifiable structure/activity relationship (SAR) between the "hit" and the CCR11 polypeptide.

There are a number of different libraries used for the identification of small molecule modulators, including, (1) chemical libraries, (2) natural product libraries, and (3) combinatorial libraries comprised of random peptides, oligonucleotides or organic molecules.

Chemical libraries consist of structural analogs of known compounds or compounds that are identified as "hits" or "leads" via natural product screening. Natural product libraries are collections from microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: (1) fermentation and extraction of broths from soil, plant or marine microorganisms or (2) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. For a review, see *Science*, *282*:63-68 (1998). Combinatorial libraries are composed of large numbers of peptides, oligonucleotides or organic compounds as a mixture. They are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning or proprietary synthetic methods. Of interest are peptide and oligonucleotide combinatorial libraries. Still other libraries of interest include peptide, protein,

peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. For a review of combinatorial chemistry and libraries created therefrom, see Myers, *Curr. Opin. Biotechnol.*, *8*:701-707 (1997).

Identification of modulators through use of the various libraries described herein permits modification of the candidate "hit" (or "lead") to optimize the capacity of the "hit" to modulate activity.

The invention also provides compositions comprising modulators of CCR11. Preferably, the compositions are pharmaceutical compositions. The pharmaceutical compositions optionally may include pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluents that serve as pharmaceutical vehicles, excipients, or media. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, gum acacia, calcium phosphate, mineral oil, cocoa butter, and oil of theobroma.

The pharmaceutical compositions can be packaged in forms convenient for delivery. The compositions can be enclosed within a capsule, sachet, cachet, gelatin, paper, or other container. These delivery forms are preferred when compatible with entry of the immunogenic composition into the recipient organism and, particularly, when the immunogenic composition is being delivered in unit dose form. The dosage units can be packaged, *e.g.*, in tablets, capsules, suppositories or cachets.

The pharmaceutical compositions may be introduced into the subject to be treated by any conventional method including, *e.g.*, by intravenous, intradermal, intramuscular, intramammary, intraperitoneal, intrathecal, intraocular, retrobulbar, intrapulmonary (*e.g.*, aerosolized drug solutions) or subcutaneous injection (including depot administration for long term release) ; by oral, sublingual, nasal, anal, vaginal, or transdermal delivery; or by surgical implantation, *e.g.*, embedded under the splenic capsule, in the brain, or in the cornea. The treatment may consist of a single dose or a plurality of doses over a period of time.

Compositions are generally administered in doses ranging from 1 μg/kg to 100 mg/kg per day, preferably at doses ranging from 0.1 mg/kg to 50 mg/kg per day, and more preferably at doses ranging from 1 to 20 mg/kg/day. The composition may be administered by an initial bolus followed by a continuous infusion to maintain therapeutic circulating levels of drug product. Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual patient. The frequency of dosing will depend on the pharmacokinetic parameters of the agents and the route of administration. The optimal pharmaceutical formulation will be determined by one skilled in the art depending upon the route of administration and desired dosage. See for example, *Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712, the disclosure of which is hereby incorporated by reference. Such formulations may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose may be calculated according to body weight, body surface area or organ size. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein. Appropriate dosages may be ascertained through use of established assays for determining blood levels dosages in conjunction with appropriate dose-response data. The final dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, *e.g.* the drug's specific activity, the severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. As studies are conducted, further information will emerge regarding the appropriate dosage levels and duration of treatment for various diseases and conditions.

It will be appreciated that the pharmaceutical compositions and treatment methods of the invention may be useful in the fields of human medicine and veterinary medicine. Thus, the subject to be treated may be a mammal, preferably

human, or other animals. For veterinary purposes, subjects include, for example, farm animals including cows, sheep, pigs, horses, and goats, companion animals such as dogs and cats; exotic and/or zoo animals; laboratory animals including mice, rats, rabbits, guinea pigs, and hamsters; and poultry such as chickens, turkeys, ducks and geese.

Association of CCR11 with chemokine activity and therefore with host infection response and the inflammatory response makes compositions of the invention, including for example, a CCR11 polypeptide, an inhibitor thereof, an antibody, or other modulator of CCR11 expression or biological activity, useful for treating any of a number of conditions. For example, aberrant CCR11 activity can be associated with many inflammatory diseases, for example, rheumatoid arthritis, inflammatory bowel disease, and asthma. Still other conditions possibly associated with chemokine activity include angiogenesis, artherosclerosis vascular association diseases which may include but are not limited to: hypertension, angina pectoris, cardiac arrhythmias, left ventricular diastolic dysfunction, Raynaud's phenomenon, migraine, preterm labor, esophageal spasm, ischemic stroke, subarachnoid hemorrhage, myocardial infarction, congestive heart failure, endometriosis, vasospasm, retinopathy, nephropathy, and pulmonary vascular disease.

Based on the information provided herein, the invention further comprises a method of modulating a biological activity mediated by the Bonzo chemokine receptor. In particular, the invention includes methods for inhibiting or promoting binding between Bonzo and its ligands including SLC, MCP-4, ELC, and MCP-2.

The invention also includes a method for identifying a specific binding partner of a Bonzo polypeptide. For example, methods are enabled that comprise steps of contacting the Bonzo polypeptide with a test compound under conditions that permit binding between the Bonzo polypeptide and the test compound; detecting binding of the Bonzo polypeptide with the test compound; and identifying the compound as a specific binding partner of the Bonzo polypeptide.

Alternatively, the invention provides a method for screening a plurality of test compounds for binding with a Bonzo polypeptide, comprising steps of

contacting the Bonzo polypeptide with each of a plurality of test compounds for a time sufficient to allow binding under suitable conditions; and detecting binding of the Bonzo polypeptide to each of the plurality of test compounds, thereby identifying the test compound as a compound that binds the Bonzo polypeptide. These methods advantageously employ competition between the test compound and a known ligand of Bonzo to provide a relative measure of affinity and activity for the test compound. Thus, the invention enables high throughput screening of libraries of chemical and biological substances to identify compounds that modulate Bonzo binding to its ligands. Accordingly, the invention also includes compounds or substances that compete with known ligands of Bonzo and that are identified using the described methods.

Moreover, the invention provides methods for identifying modulators of a biological activity of Bonzo mediated by its ligands. For example, the invention enables a method for identifying a modulator of chemotaxis activity mediated by a Bonzo polypeptide, comprising steps of admixing the Bonzo polypeptide with a test compound; incubating the admixture in the presence of a known binding partner of the Bonzo polypeptide; measuring an amount of binding of the known binding partner with the Bonzo polypeptide; and comparing the measured amount of binding with an amount of binding of the known binding partner with the Bonzo polypeptide when incubated in the absence of the test compound, wherein a comparative difference in the amounts of binding in the presence and absence of the test compound indicates that the test compound is a modulator of chemotaxis activity mediated by the Bonzo polypeptide.

The information provided herein identifies specific ligands for Bonzo including SLC, MCP-4, ELC, and MCP-2, Accordingly, there are also contemplated methods of using these ligands in the treatment of medical disorders in which the activity of Bonzo is implicated. Thus, pharmaceutical compositions comprising effective amounts of one or more of these chemokines may be administered to a vertebrate animal subject, *e.g.*, a human, suffering from or susceptible to a disorder mediated by or characterized by a biological activity of Bonzo.

There is also provided a method for modulating Bonzo binding to a binding partner thereof, comprising the step of contacting Bonzo or the binding partner thereof with a modulator of Bonzo binding to the binding partner. Similarly, there is provided a method for modulating a biological activity of Bonzo, comprising contacting Bonzo with a modulator of its activity. Such methods may be performed *in vitro*, but are preferably performed *in vivo, e.g.*, in medical treatment of disease in vertebrates such as humans and other animals. Use of these chemokines as probes in *in vitro* assays, *e.g.*, diagnostic techniques is also contemplated.

Accordingly, the invention enables treatment of diseases that are mediated by or characterized by activity of cells that express Bonzo, *e.g.*, T cells. For example, the invention permits the use of SLC, MCP-4, ELC, or MCP-2 or modulators of Bonzo binding to any of these chemokines for treatment of T cell-mediated diseases in humans. Such modulators include, for example, small organic molecules, peptides, and peptidomimetics that compete with any of the ligands of Bonzo, and antibodies that inhibit or prevent binding between Bonzo and its ligands.

The invention is illustrated by the following examples, which are intended as illustrative but not limiting. Example 1 relates to cloning and expression of CCR11. Example 2 describes chromosomal localization of a CCR11 gene. Example 3 describes Northern analysis of CCR11 expression. Example 4 relates to functional responses of CCR11 transfectants. Example 5 describes a competition assay designed to measure specificity of binding to CCR11. Example 6 relates to production of anti-CCR11 polypeptide antibodies. Example 7 describes assays to identify ligands and/or modulators of CCR11. Example 8 describes a chemoselection assay used to identify ligands capable of stimulating chemotaxis through the receptor Bonzo.

EXAMPLE 1
CLONING AND EXPRESSION OF CCR11 DNA

A. Database search

The orphan receptor PPR1 was originally isolated from bovine papillary tissue in a search for gustatory receptors. See Matsuoka et al., *Biochem Biophys Res Commun, 194*:504-11 (1993). However, the expression of PPR1 appears to be higher in lung than in tongue. In addition, PPR1 shares more similarity with chemokine receptors than gustatory or olfactory receptors. Because of this similarity, a human homolog of PPR1 was isolated and its ability to function as a chemokine receptor was examined.

The GenBank Expressed Sequence Tag (EST) database was searched with the NCBI program BLASTP 2.0.10 using the GPCR bovine PPR1 cDNA sequence described in Matsuoka, *et al.*, *Biochem Biophys Res Commun*, *194*:504-511 (1993) as the query sequence. (SEQ ID NO: 3). The bovine amino acid sequence encoded by SEQ ID NO: 3 and having 35% homology to human chemokine receptors CCR7 and CCR9 is set forth in SEQ ID NO: 4.

The search identified three human ESTs with high homology to the bovine sequence: H67224, AA215577, and AI131555 with 87%, 79% and 74% identity, respectively. IMAGE clone 238667 (H67224) was obtained from Research Genetics (Huntsville, AL) and the entire insert was sequenced using standard techniques. (SEQ ID NO: 5) The 831 base pair insert included a long open reading frame encoding a polypeptide of 270 amino acids that was 86% identical to the bovine protein. When aligned with the bovine protein, the human protein sequence was determined to be missing the first 80 amino acids (including the extracellular amino terminus, the first transmembrane domain and the first intracellular loop). Therefore, additional cDNA libraries were screened to obtain a complete open reading frame, which was ultimately called CCR11.

Three human cDNA libraries were hybridized with a probe derived from the H67224 insert sequence. The probe was prepared by Polymerase Chain Reaction (PCR) amplification of a CCR11 fragment from human genomic DNA with a primer containing (the complement of) nucleotides 130-151 of SEQ ID NO: 5 and a

primer containing nucleotides 590-610 of SEQ ID NO: 5. The PCR reaction was performed with an initial denaturing at 94°C for four minutes followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The sequence of the resulting probe is set forth in SEQ ID NO: 6. Libraries from small intestine (Stratagene, La Jolla, CA), macrophage as described by Chantry, *et al.*, *J Leukoc Biol*, *64*:49-54 (1998), and peripheral blood mononuclear cells (PBMC) were probed. Hybridization conditions were as follows: Overnight incubation at 42°C in 750 mM sodium chloride (NaCl), 75 mM sodium citrate, 50 mM sodium phosphate, 5 mM EDTA, 0.05% FICOLL, 0.05% polyvinylpyrrolidone (PVP), 0.05% bovine serum albumin, 50% formamide, 100µg/ml sheared salmon sperm DNA and 0.1% sodium dodecyl sulfate (SDS). The filters were washed several times at 50°C in 30 mM sodium chloride, 3 mM sodium citrate, 0.1% sodium dodecyl sulfate.

Over one million clones were examined in each library. No clones were found in the macrophage library. A single clone was identified in the peripheral blood mononuclear cell (PBMC) library that was 1388 base pairs in length and lacked 188 base pairs of the amino terminal coding sequence (SEQ ID NO: 7). Five clones were isolated from the small intestine cDNA library, ranging in size from 131 to 1,153 base pairs (SEQ ID NOS: 8, 9, 10, 11, and 12). Nucleotide sequences of the small intestine clones were aligned and a consensus sequence was deduced. The resulting consensus cDNA sequence (SEQ ID NO: 13), when aligned with the bovine PPR1 sequence, contained the entire open reading frame except for 14 base pairs at the 5′ end of the coding sequence.

Four nucleotide differences were observed between the PBMC cDNA and the small intestine consensus sequence, one of which results in an amino acid difference: the PBMC clone has an asparagine at amino acid 143 whereas the small intestine clones encode a lysine at this position. The significance of this allelic difference is not known at this time.

To determine the 5′ coding region, a human genomic P1 library (Genome Systems, St. Louis, MO) was screened by PCR (35 cycles of 94°C for one minute followed by 68°C for one minute) using the primers described above to isolate a CCR11 genomic clone (SEQ ID NO: 14). The genomic clone was sequenced and

provided the 5' coding sequence of CCR11. The genomic sequence presented in SEQ ID NO: 14 appears to contain no intervening sequences since it has contiguous homology with the bovine cDNA sequence throughout the coding region and for 263 nucleotides upstream of the start codon. The genomic sequence was identical to the small intestine allele with lysine at position 143. A DNA and deduced amino acid sequence for CCR11 are set forth in SEQ ID NOs: 1 and 2, respectively.

Human CCR11 is 86% identical to bovine PPR1 at the amino acid level and 88% identical at the nucleotide level. This high degree of similarity is consistent with other chemokine receptor genes when compared across mammalian species. CCR11 contains potential N-linked glycosylation sites, two in the amino terminal extracellular domain and one in the third extracellular loop. Furthermore, CCR11 contains single cysteine residues in each of the four predicted extracellular domains. CCR11 shares 28-36% identity with other human chemokine receptors. The receptor with the highest homology to CCR11 is CCR7 (about 36% identical at the amino acid level), followed by CCR6 and CCR9 (each about 33% identical to CCR11). CCR11 is less homologous to other members of the GPCR superfamily, the next closest being lipid mediator receptors (platelet activating factor receptor, about 24%; leukotriene B4 receptor, about 24%) and the chemotactic peptide receptor (fMet-Leu-Phe receptor, about 23%).

B. Expression of CCR11 in Murine pre-B cell line L1.2

The CCR11 coding region (SEQ ID NO: 1) was amplified from the P1 clone with primers 5'-GCCCAAGCTTGCCACCATGGCTTTGGAACAGAACCAGTCAAC (SEQ ID NO: 15) and 5'-CTAGTCTAGAGTATCCAGGCAAAAGGCAGAGCAG (SEQ ID NO: 16) which included HindIII and Xbal cloning sites (underlined). This amplified fragment was inserted into pNEF6, a vector containing the Chinese hamster Elongation Factor-1α gene promoter and neomycin resistance gene. The coding region of CCR11 in the resulting expression vector was sequenced to ensure no mutations were introduced by PCR.

The murine pre-B cell line L1.2 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The expression construct was transfected into mouse pre-B L1.2 cells by electroporation with 10 µg of plasmid at 250V, 960 microfarads, 72 ohm resistance using a Gene Pulser® (Bio-Rad, Hercules, CA). Transfectants were selected and expanded in 800 µl/ml G418. These cells were used in a chemotaxis assay against a panel of chemokines in order to identify ligands for CCR11 and to isolate CCR11 expressing transfectants.

C. Expression of CCR11 in HEK293 Cells

The CCR11 expression construct described in Part B was stably transfected into HEK293 cells using the SuperFect transfection reagent (Qiagen). In brief, 15 µg of plasmid DNA was mixed with 60 µl of SuperFect reagent in a total volume of 360 µl. After a 10 minutes incubation at room temperature, 3 ml of growth media (MEM with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 µg/ml Pen-Strep, 2 mM L-glutamine) was added to the transfection complex and the whole mixture spread on top of washed HEK 293 cells in a 10 cm dish. After a 2-hour incubation at 37°C, the cells were washed extensively and then incubated with growth media overnight. The next day, the cells were split 1:10 into five 15 cm dishes, and 800 µg/ml G418 was added to the growth media to select for cells that had been successfully transfected with the plasmid. After two weeks, 15 stably transfected clones were isolated and expanded into larger cultures.

The amount of CCR11 mRNA expressed by each clone was determined by Northern blot analysis. RNA was extracted from the cells using RNA STAT-60 (TelTest). A 10 µg sample of RNA from each cell line was electrophoresed on an agarose gel containing 6.6% formaldehyde. RNA was transferred from the gel to a nitrocellulose filter overnight and then probed with a radiolabeled coding fragment of CCR11 DNA. The clone with the highest level of CCR11 mRNA expression was chosen for further analysis.

EXAMPLE 2
CHROMOSOMAL LOCALIZATION STUDY

Many GPCR genes are clustered in the human genome. Indeed, the genes for the majority of the CC chemokine receptors are encoded at 3p21. Samson, et al., *Genomics*, *36*:522-526 (1996); Raport, *et al.*, *J. Biol. Chem.*, *271*:17161-17166 (1996). Since clusters of genes are generally functionally related, the chromosomal location of the CCR11 gene was identified in order to help determine its function.

The approximately 90 kilo-basepair genomic P1 clone containing the human CCR11 gene was labeled with digoxigenin dUTP by nick translation and used as a probe for fluorescence *in situ* hybridization of human chromosomes (Genome Systems, Inc., St. Louis, MO). The labeled probe was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. Reactions were carried out in the presence of sheared human DNA in 50% formamide, 10% dextran sulfate, 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate. Hybridization signals were detected by treating slides with fluoresceinated antidigoxigenin antibodies followed by counter staining with 4,6-diamidino-2-phenylindole. Initial hybridization resulted in specific labeling of the middle long arm of a group A chromosome believed to be chromosome 3 on the basis of size, morphology, and banding pattern.

A biotin labeled genomic probe that was specific for the centromere of chromosome 3 was then co-hybridized with the CCR11 probe and detected with Texas Red avidin. This resulted in specific labeling of the centromere of chromosome 3 in red and the middle long arm of chromosome 3 in green. Measurement of ten specifically labeled chromosomes demonstrated that the CCR11 gene is located at a position that is 42% of the distance from the centromere to the telomere of chromosome arm 3q, an area which corresponds to 3q22. A total of eighty metaphase cells were analyzed, with seventy-two exhibiting specific labeling.

As noted above, most other CC chemokine receptor genes are on chromosome 3 but at 3p21, so CCR11 is significantly separated from this gene cluster. This suggests that CCR11 is more distantly related to most CC chemokine receptors. FIG. 1 is a dendrogram analysis which illustrates the similarity of the deduced amino acid sequence of CCR11 with previously identified CC chemokine receptors. The CCR11 gene is closer on chromosome 3 to the orphan receptor

GPR15, also known as BOB, which has been localized at 3q11.2-13.1 as described in Heiber, *et al.*, *Genomics*, *32:*462-465 (1996).

EXAMPLE 3
NORTHERN BLOT ANALYSIS

The expression pattern of CCR11 in various human tissues was examined by Northern blot analysis. Human multi-tissue Northern blots were purchased from Clontech (Palo Alto, CA) and hybridized as described in Raport, et al., *J. Biol. Chem.*, *271:*17161-17166 (1996). A gel-purified cDNA fragment containing 1388 base pairs which included the entire of the coding region of human CCR11 (nucleotides 1 to 1388 of SEQ ID NO: 7) was used as a hybridization probe.

The nylon blots were hybridized at 42°C in 0.75 M sodium chloride, 75 mM sodium citrate, 50 mM sodium phosphate (pH 6.5), 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 1% sodium dodecyl sulfate, 100 µg/ml sheared salmon sperm DNA and 50% formamide. The blots were washed extensively at 50°C in 30 mM sodium chloride, 3 mM sodium citrate, 0.1% sodium dodecyl sulfate.

In human tissues, CCR11 mRNA was expressed most abundantly in human heart, small intestine and lung. Lower levels of expression were observed in kidney, liver and colon. The size of the primary transcript was approximately 2000 bases, which corresponds well with the cDNA size. The most abundant transcript in heart appeared to be of greater size than that seen in other tissues and perhaps represents an alternatively spliced transcript.

EXAMPLE 4
MIGRATION ASSAY TO CHARACTERIZE CCR11 FUNCTIONAL RESPONSE

In order to determine if CCR11 functions as a chemokine receptor, murine L1.2 cells that had been transfected with CCR11 as described in Example 1 above were tested for chemotactic response to a panel of twenty-nine human chemokines. This panel included MIP-1α, MIP-1β, MCP-3, MCP-4, ELC, SLC,

NAP-2, ENA-78, HCC-1, fractalkine, lymphotactin, SDF-1, 1-309, IL-8, IP-10, HCC-4, LKN-1/MIP-5 (all purchased from R & D Systems, Minneapolis MN); MCP-1, PARC, MDC, TARC, and eotaxin (all purchased from Gryphon Sciences, So. San Francisco, CA); and RANTES, MCP-2, LARC, TECK, PF4, MGSA, and MIG (all purchased from Peprotech, Rocky Hill, NJ).

Approximately 10^6 murine L1.2 cells suspended in 0.1 ml of RPMI 1640 medium with 0.5% (endotoxin reduced) BSA (Intergen, Purchase, NY) were loaded in the upper wells of a Transwell® chamber (3-μm pore size, 6.5 mm diameter, Costar, Cambridge, MA). For the initial experiments, each of the twenty-three human chemokines in the panel were tested at 1 nM and 10 nM because these concentrations are often the optimal for other chemokine-chemokine receptor interactions. The panel chemokines were added to the lower wells in a volume of 0.6 ml. After four hours at 37°C, cells that had migrated to the lower chamber were collected and counted by visual inspection. Untransfected L1.2 cells were used as a control.

In initial experiments, the most significant migration of CCR11 transfectants was observed toward MCP-4, with some chemotaxis also observed toward MCP-2 and MCP-1. No other chemokines induced significant cell migration.

The CCR11 transfectants that migrated towards MCP-4 were harvested, cloned by limiting dilution, and expanded by growth in RPMI media with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum for further studies.

For example, the CCR11 transfectants were tested for calcium mobilization in response to ligand stimulation. Murine cells were resuspended at 3×10^6 cells/ml in RPMI media with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum. Cells were incubated with 1 μM fura-2-AM (Molecular Probes, Eugene OR) at room temperature for 30 minutes in the dark. After washing, cells were resuspended at 2×10^6 cells/ml. To measure intracellular calcium, cells in 2 ml were placed in a quartz cuvette in a SLM Aminco-Bowman Series 2 Luminescence Spectrometer. Fluorescence was monitored at 340 nm (excitation wavelength 1), 380 nm (excitation wavelength 2), and 510 nm (emission wavelength). Chemokines were added at 100 nM final

concentration. Small but significant calcium flux was observed when transfectants were stimulated with MCP-4. No calcium flux was observed in response to MCP-1 or MCP-2 stimulation.

However, further Northern blot study of these initial transfected L1.2 cells indicated that endogenous murine CCR2 expression was upregulated. Since cells transfected with recombinant murine CCR2 have a ligand recognition pattern that includes these same MCP chemokines, it was concluded that the observed chemotaxis was probably due to murine CCR2.

Subsequent transfection experiments produced L1.2 and HEK293 expressing high levels of CR11, as confirmed by CCR11-specific antiserum. Both cell lines demonstrate binding to the chemokines ELC (EBII Ligand Chemokine, also known as MIP-3β, Genbank Acc. No. Q99731), SLC (Secondary Lymphoid tissue Chemokine, GENBANK Acc. No. O00585), TECK (Thymus-Exporessed ChemoKine, Genbank Acc. No. O15444), as also result reported by Gosling *et al., J. Immunol., 164*: 2851-2856 (2000).

EXAMPLE 5
RECEPTOR BINDING ASSAY

Binding of putative ligands to CCR11-transfected L1.2 or HEK293 cells can be examined by competition assay. For example, 5 x 10^5 CCR11 transfected cells are incubated for one hour at room temperature with 0.1 nM ^{125}I-labeled chemokine ligand (ELC, SLC, TECK) in the presence or absence of various concentrations of chemokines in 200 µl of binding buffer (25 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1% BSA). Following incubation, cells are transferred to polyethyleneimine-coated GF-B 96 well plates and washed three times in wash buffer (25 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5 M NaCl).

Scintillant is added to each well, and bound ligand is quantified using a Wallac 1450 Microbeta Liquid Scintillation Counter (Gaithersburg, MD). Binding competition curves are fitted using a four parameter logistic equation (GraphPad Prism®, GraphPad Software, San Diego, CA).

HEK293/CCR11 cells prepared as described in Example 1 and expressing high levels of CCR11 mRNA were tested in competition binding assays using iodinated chemokines, essentially as described in the preceding paragraphs. ^{125}Iodinated MCP-4 was not observed to bind above background levels. ^{125}Iodinated ELC readily bound to these CCR11 expressing cells. As shown in Figure 2, ^{125}I-ELC binding could be displaced with increasing concentrations of unlabeled ELC, SLC and TECK, but not with MCP-4.

EXAMPLE 6
ANTIBODIES TO CCR11

Standard techniques are employed to generate polyclonal or monoclonal antibodies to CCR11 and to generate useful antigen-binding fragments thereof or variants thereof, including "humanized" variants. Such protocols can be found, for example in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual* Second Edition, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989); Harlow, *et al.* (Eds), *Antibodies A Laboratory Manual*; Cold Spring Harbor Labor Laboratory, Cold Spring Harbor, NY (1988). In one embodiment, recombinant CCR11 polypeptides (or cells or cell membranes containing such polypeptides) are used as antigens to generate the antibodies. In another embodiment, one or more peptides having amino acid sequences corresponding to an immunogenic portion of CCR11 (*e.g.*, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acids) are used as antigens. Peptides corresponding to extracellular portions of CCR11, especially hydrophyllic extracellular portions, are preferred. The antigen may be mixed with an adjuvant or linked to a hapten to enhance antibody production.

A. Polyclonal or Monoclonal Antibodies

In one exemplary protocol, recombinant CCR11 or a synthetic fragment thereof is used to immunize a mouse for generation of monoclonal antibodies or a rabbit or other larger mammal for generation of polyclonal antibodies. To increase antigenicity, peptides are conjugated to Keyhole Limpet Hemocyanine (Pierce) according to the manufacturer's recommendations. For an initial injection, the antigen is emulsified with Freund's Complete Adjuvant and injected

subcutaneously. At intervals of two to three weeks, additional aliquots of CCR11 antigen are emulsified with Freund's Complete Adjuvant and injected subcutaneously. Prior to the final booster injection, a serum sample is taken from the immunized mice and assayed by western blot to confirm the presence of antibodies that immunoreact with CCR11. Serum from the immunized animals may be used as polyclonal antisera or used to isolate polyclonal antibodies that recognize CCR11. Alternatively, the mice are sacrificed and their spleens removed for generation of monoclonal antibodies by methods well known in the art.

B. Humanization of anti-CCR11 monoclonal antibodies

The expression pattern of CCR11 as reported herein and the proven track record of GPCRs as targets for therapeutic intervention suggest therapeutic indications for CCR11 inhibitors. CCR11 neutralizing antibodies comprise one class of therapeutics useful as CCR11 inhibitors. Humanizing the monoclonal antibodies specific for CCR11 improves their serum half-life and renders them less immunogenic in human hosts. The principles of humanization have been described in the literature and are facilitated by the modular arrangement of antibody proteins. To minimize the possibility to binding complement, a humanized antibody of the IgG_4 isotype is preferred.

For example, a level of humanization is achieved by generating chimeric antibodies comprising the variable domains of non-human antibody proteins of interest with the constant domains of human antibody molecules. See, *e.g.*, Morrison and Oi, *Adv Immunol, 44:65-92* (1989). The variable domains of CCR11 neutralizing anti-CCR11 antibodies are cloned from the genomic DNA of a B-cell hybridoma or from cDNA generated from mRNA isolated from the hybridoma of interest. The V region gene fragments are linked to exons encoding human antibody constant domains and the resultant construct is expressed in suitable mammalian host cells (*e.g.*, myeloma or CHO cells).

To achieve an even greater level of humanization, only those portions of the variable region gene fragments that encode antigen-binding complementarity determining regions (CDR) of the non-human monoclonal antibody genes are cloned

into human antibody sequences. See, *e.g.*, Jones *et al.*, *Nature, 321:*522-525 (1986); Riechmann, et al., *Nature, 332:*323-327 (1988); Verhoeyen, *et al.*, *Science, 239:*1534-1536 (1988); and Tempest, *et al.*, *Bio/Technology, 9:*266-271 (1991). If necessary, the β-sheet framework of the human antibody surrounding the CDR3 regions also is modified to more closely mirror the three dimensional structure of the antigen binding domain of the original monoclonal antibody. See Kettleborough, *et al.*, *Protein Engin., 4:*773-783 (1991) and Foote, *et al.*, *J. Mol. Biol., 224:*487-499 (1992).

In an alternative approach, the surface of a non-human monoclonal antibody of interest is humanized by altering selected surface residues of the non-human antibody, *e.g.*, by site directed mutagenesis, while retaining all of the interior and contacting residues of the non-human antibody. Padlan, *Molecular Immunol., 28(4/5):*489-498 (1991).

The foregoing approaches are employed using CCR11 neutralizing anti-CCR11 monoclonal antibodies and the cell lines that produce them to generate humanized CCR11 neutralizing antibodies useful as therapeutics to treat or palliate conditions wherein CCR11 expression or ligand-mediated CCR11 signaling is detrimental.

C. Human CCR11 Neutralizing Antibodies from Phage Display

Human CCR11-neutralizing antibodies are generated by phage display techniques such as those described in Aujame *et al.*, *Human Antibodies, 8(4):*155-168 (1997); Hoogenboom, *TIBTECH, 15:*62-70 (1997); and Rader *et al.*, *Curr. Opin. Biotechnol., 8:*503-508 (1997), all of which are incorporated herein by reference. For example, antibody variable regions in the form of Fab fragments or linked single chain Fv fragments are fused to the amino terminus of filamentous phage minor coat protein pIII. Expression of the fusion protein and incorporation thereof into the mature phage coat results in phage particles that present an antibody on their surface and contain the genetic material encoding the antibody. A phage library comprising such constructs is expressed in bacteria, and the library is panned (screened) for CCR11-specific phage-antibodies using labelled or immobilized CCR11 as antigen-probe.

D. Human CCR11 Neutralizing Antibodies from Transgenic Mice

Human CCR11-neutralizing antibodies are generated in transgenic mice essentially as described in Bruggemann and Neuberger, *Immunol. Today, 17(8):*391-97 (1996) and Bruggemann and Taussig, *Curr. Opin. Biotechnol., 8:*455-58 (1997). Transgenic mice carrying human V-gene segments in germline configuration and that express these transgenes in their lymphoid tissue are immunized with a CCR11 composition using conventional immunization protocols. Hybridomas are generated using B cells from the immunized mice using conventional protocols and screened to identify hybridomas secreting anti-CCR11 human antibodies (*e.g.*, as described above).

E. Fully Human Antibodies

Fully human antibodies are especially preferred for therapeutic use in humans, but they are typically difficult to produce. For example, when the immunogen is a human self-antigen, a human will typically not produce any immune response to the antigen. Methods for making fully human antibodies have been developed and are known in the art. Accordingly, fully human antibodies can be produced by using an immunogenic CCR11 polypeptide to immunize an animal (*e.g.*, mouse) that has been transgenically modified to express at least a significant fraction of the human repertoire of immunoglobulin genes. See *e.g.*, Bruggemann *et al.*, *Immunol Today, 17*:391-397 (1996).

EXAMPLE 7
ASSAYS TO IDENTIFY MODULATORS OF CCR11 ACTIVITY

Set forth below are assays for identifying modulators that block, inhibit or stimulate CCR11. The modulators that can be identified by these assays include, but are not limited to, natural ligand compounds of the receptor; synthetic analogs and derivatives of natural ligands; antibodies, antibody fragments, and/or antibody-like compounds derived from natural antibodies or from antibody-like combinatorial libraries; and/or synthetic chemical compounds. All modulators that bind CCR11 are useful for identifying CCR11 in tissue samples (*e.g.*, for diagnostic purposes). Modulators are useful for stimulating and inhibiting CCR11 activity and for treating

disease states characterized by abnormal levels of CCR11. CCR11 binding molecules also may be used to deliver a therapeutic compound or a label to cells that express CCR11 (*e.g.*, by attaching the compound or label to the binding molecule). The assays may be performed using single putative modulators, and/or may be performed using a known stimulatory modulator in combination with candidate inhibitory modulators (or *visa versa*).

A. Direct Assay for Ligands

Detectably labeled test compounds are exposed to membrane preparations presenting chemokine receptors in a functional conformation. For example, HEK-293 cells, or tissue culture cells, are transfected with an expression vehicle encoding CCR11. A membrane preparation is then made from the transfected cells expressing CCR11. The membrane preparation is exposed to ^{125}I-labeled test compounds (*e.g.*, chemokines) and incubated under suitable conditions (*e.g.*, ten minutes at 37°C). The membranes, with any bound test compounds, are then collected on a filter by vacuum filtration and washed to remove unbound test compounds. The radioactivity associated with the bound test compound is then quantitated by subjecting the filters to liquid scintillation spectrophotometry. The specificity of test compound binding may be confirmed by repeating the assay in the presence of increasing quantities of unlabeled test compound and noting the level of competition for binding to CCR11.

This binding assay can also identify modulators of CCR11. The previously described binding assay may be performed with the following modifications. In addition to detectably labeled test compound, a potential modulator is exposed to the membrane preparation. An increased level of membrane-associated label indicates the potential modulator is a stimulatory modulator; a decreased level of membrane-associated label indicates the potential modulator is an inhibitor of CCR11 binding.

B. Indirect Assay for Ligands

I. GTP Hydrolysis Assay

As reviewed in Linder, et al., *Sci. Am.*, *267*:56-65 (1992), during signal transduction, an activated receptor interacts with a G protein, in turn activating the G protein. The G protein is activated by exchanging GDP for GTP. Subsequent hydrolysis of the G protein-bound GTP deactivates the G protein. One assay for G protein activity therefore monitors the release of $^{32}P_i$ from [γ-^{32}P]-GTP. For example, approximately 5 X 10^7 HEK-293 cells harboring plasmids of the invention are grown in MEM + 10% FCS. The growth medium is supplemented with 5 mCi/ml [^{32}P]-sodium phosphate for 2 hours to uniformly label nucleotide pools. The cells are subsequently washed in a low phosphate isotonic buffer. One aliquot of washed cells is then exposed to a test compound while a second aliquot of cells is treated similarly, but without exposure to the test compound. Following an incubation period (*e.g.*, ten minutes), cells are pelleted, lysed and nucleotide compounds fractionated using thin layer chromatography developed with 1 M LiCl. Labeled GTP and GDP are then quantitated by autoradiographic techniques that are standard in the art. Relatively high levels of ^{32}P-labeled GDP identify test compounds as ligands.

This type of GTP hydrolysis assay is also useful for identifying modulators of CCR11 binding. The aforementioned assay is performed in the presence of a potential modulator. An intensified signal resulting from a relative increase in GTP hydrolysis, producing ^{32}P-labeled GDP, indicates a relative increase in receptor activity. The intensified signal therefore identifies the potential modulator as a stimulatory modulator. Conversely, a diminished relative signal for ^{32}P-labeled GDP, indicative of decreased receptor activity, identifies the potential modulator as an inhibitor of CCR11 binding.

II. [^{35}S]GTPγS Binding Assay

Because G protein coupled receptors signal through intracellular "G proteins" whose activity involves GTP/GDP binding and hydrolysis, measurement of binding of the non-hydrolyzable GTP analog

[^{35}S]GTPγS in the presence and absence of putative modulators provides another indicator of modulator activity. See, *e.g.*, Kowal, *et al.*, *Neuropharmacology*, *37*:179-87 (1998).

In one exemplary assay, cells stably transfected with a CCR11 expression vector are grown in 10 cm dishes to subconfluence, rinsed once with 5 ml of ice cold Ca^{2+}/Mg^{2+} free PBS, and scraped into 5 ml of the same buffer. Cells are pelleted by centrifugation (500 x g, 5 minutes), resuspended in 25 mM Tris, 5 mM EDTA, 5 mM EGTA, pH 7.5 (TEE), and frozen in liquid nitrogen. After thawing, the cells are homogenized using a dounce (one ml TEE per plate of cells), and centrifuged at 1,000 x g for 5 minutes to remove nuclei and unbroken cells.

The homogenate supernatant is centrifuged at 20,000 x g for 20 minutes to isolate the membrane fraction, and the membrane pellet is washed once with TEE and resuspended in binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA). The resuspended membranes can be frozen in liquid nitrogen and stored at -70°C until use.

On the day of the assay, frozen homogenates of cell homogenate are thawed and centrifuged as 48000 g for 10 minutes. The supernatant is discarded and the resultant pellet is resuspended with 20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4 buffer to a final protein concentration of 250 µg/ml. Using 12 x 75 mm disposable glass test tubes, buffer, test compound, GDP and 25 µg protein are combined to achieve a volume of 900 µl. Assay tubes are preincubated at 30°C for 20 minutes followed by 15 minutes on ice. [^{35}S]GTPγS is added to each assay tube (final concentration of 0.1 nM) such that the total incubation volume is 1 ml. All assay tubes are incubated at 30°C for 30 minutes.

The incubation is terminated by addition of 3 ml of ice cold buffer to each test tube followed by rapid filtration over filter paper using a 48-well cell harvester. The filters are then rinsed three times with 3 ml aliquots of ice cold buffer. Samples are counted using conventional liquid scintillation spectroscopy.

An increase in the amount of the non-hydrolyzable GTP analog in the presence of the putative modulator indicates that the putative modulator stimulates CCR11. A decrease in the amount of the non-hydrolyzable GTP analog in the

presence of a putative modulator indicates that the putative modulator is an inhibitor of CCR11.

III. cAMP Assays

In one type of assay, levels of cyclic adenosine monophosphate (cAMP) are measured in CCR11-transfected cells that have been exposed to candidate modulator compounds. The binding of the candidate modulator compound to CCR11 triggers the exchange of GTP for bound GDP; the CCR11 receptor complex binds to the G protein which induces the release of bound GDP and allows the GTP to enter. The α subunit of the G protein disassociates from the β subunit and activates adenylate cyclase. Protocols for cAMP assays have been described in the literature. See, *e.g.*, Sutherland *et al., Circulation, 37:*279 (1968); Frandsen, E.K. and Krishna, G, *Life Sciences, 18:*529-541 (1976); Dooley *et al., Journal of Pharmacology and Experimental Therapeutics, 283 (2):*735-41 (1997); and George *et al., Journal of Biomolecular Screening, 2 (4):*235-40 (1997).

Briefly, the CCR11 coding sequence (*e.g.*, a cDNA or intronless genomic DNA) is subcloned into a commercial vector and transiently transfected into suitable cells using known methods, such as the transfection reagent FuGENE6 (Boehringer-Mannheim) and the transfection protocol provided in the product insert. The transfected cells are seeded into the 96-well microplates from a FlashPlate® assay kit (Dupont), which are coated with solid scintillant to which antisera to cAMP has been bound. For a control, some wells are seeded with wild type (untransfected) cells. Other wells on the plate receive various amounts of cAMP standard solution for use in creating a standard curve.

One or more test compounds are added to the cells in each well, with water and/or compound-free media/diluent serving as a control. After treatment, cAMP is allowed to accumulate in the cells for exactly 15 minutes at room temperature. The assay is terminated by the addition of lysis buffer containing [^{125}I]-labeled cAMP, and the plate is counted using a Packard Topcount™ 96-well microplate scintillation counter. Unlabeled cAMP from the lysed cells, or from standards, competes with the fixed amounts of [^{125}-I]-cAMP for antibody bound to the

plate. A standard curve is constructed and cAMP values for the unknowns are obtained by interpolation.

Changes in intracellular cAMP level of the cells in response to exposure to a test compound are indicative of CCR11 modulating activity. An elevated level of intracellular cAMP can be attributed to a ligand induced increase in receptor activity, thereby identifying a ligand. A relative reduction in the concentration of cAMP would indirectly identify an inhibitor of receptor activity.

IV. Intracellular Calcium Measurement

A common feature of signaling through known chemokine receptors is that signal transduction is associated with the release of intracellular calcium cations. Therefore, intracellular calcium assays can be employed to assay for ligands or for modulators of CCR11 activity.

In an exemplary assay, cells that have been transfected with CCR11 are grown in suitable medium to approximately 90% confluence. Cells are then washed and harvested and incubated with Fura-2 AM (Molecular Probes, Inc., Eugene, OR) for thirty minutes at room temperature. Fura-2 AM is a Ca^{++}-sensitive dye. Cells are resuspended in an appropriate buffer to a concentration of approximately 10^7 cells/ml and changes in fluorescence are monitored using a fluorescence spectrophotometer.

In another intracellular calcium assay, cells are transiently co-transfected with both a CCR11 expression construct and a construct that encodes the photoprotein apoaquorin. In the presence of the cofactor coelenterazine, apoaquorin will emit a measurable luminescence that is proportional to the amount of intracellular (cytoplasmic) free calcium. See generally Cobbold P.H. and Lee, J.A.C. "Aequorin measurements of cytoplasmic free calcium. In: McCormack J.G. and Cobbold P.H., eds., *Cellular Calcium: A Practical Approach*. Oxford:IRL Press (1991); Stables *et al., Analytical Biochemistry, 252:*115-26 (1997); and Haugland, R.P. *Handbook of Fluorescent Probes and Research Chemicals*. Sixth edition. Eugene OR: Molecular Probes (1996).]

In an exemplary assay, CCR11 is subcloned into a commercial expression vector and transiently co-transfected along with a construct that encodes

the photoprotein apoaequorin (Molecular Probes, Eugene, OR) into suitable cells using the transfection reagent FUGENE 6 (Boehringer Mannheim) and the transfection protocol provided in the product insert.

All apoaequorin assays are performed in 96-well white microtiter plates. Cells are assayed 48 hours post transfection. In order to reconstitute the holoenzyme apoaequorin, cells are incubated at 37°C in suitable medium containing 5 μM coelenterazine for three hours. Test compounds are diluted in extracellular medium (125 mM NaCl, 5 mM KCl, 2mM $MgCl_2 \cdot 6H_2O$, 0.5 mM $NaH_2PO_4 \cdot H_2O$, 5 mM $NaHCO_3$, 10 mM Hepes, 10 mM glutamine, 0.1% bovine serum albumin (BSA), pH 7.4) and added at 2X final concentration in a volume equal to that already in the assay plate (final volume, 200 μl). Luminescence is measured at room temperature for 10 seconds following addition of the test compound using the Integrate Flash mode on a Dynatech ML 3000 luminometer.

Changes in luminescence caused by the test compounds are indicative of modulatory activity. Modulators that stimulate receptors which couple to the Gq subtype of G proteins give an increase in luminescence of up to 100 fold. Inhibitory modulators will reverse this effect at receptors that are either constitutively active or activated by known stimulatory modulators.

In another assay, cells stably transfected with a CCR11 expression vector are plated at a density of 4×10^4 cells/well in Packard black-walled 96-well plates specially designed to isolate fluorescent signal to individual wells. The cells are incubated for 60 minutes at 37°C in modified Dulbecco's PBS (D-PBS) containing, 36 mg/L pyruvate and 1 g/L glucose with the addition of 1% fetal bovine serum and one of four calcium indicator dyes (Fluo-3™ AM, Fluo-4™ AM, Calcium Green™-1 AM, or Oregon Green™ 488 BAPTA-1 AM) at a concentration of 4 μM. Plates are washed once with modified D-PBS without 1% fetal bovine serum and incubated for 10 minutes at 37°C to remove residual dye from the cellular membrane. In addition, a series of washes with modified D-PBS without 1% fetal bovine serum is performed immediately prior to activation of the calcium response.

Calcium response is initiated by the addition of one or more candidate receptor modulator compounds, calcium ionophore A23187 (10 μM), or ATP (4 μM).

Fluorescence is measured by Molecular Device's FLIPR equipped with an argon laser, excitation at 488 nm, an automated 96 channel pipetter and a CCD camera. See, *e.g.*, Kuntzweiler *et al., Drug Development Research, 44(1):*14-20 (1998).

The intensity of the fluorescence is captured by the CCD camera every second for the first minute following addition of the candidate modulator, with additional readings every 5 seconds for a total time period of 5 minutes. The exposure setting of the camera is 0.4 seconds with an f-stop setting of 2 microns. Changes in fluorescence are indicative of modulation of CCR11. Stimulatory modulators give an increase in fluorescence while inhibitory modulators give a decrease in fluorescence.

V. Phospholipase C Assay

Another assay for ligands or modulators involves monitoring phospholipase C activity, as described in Hung, *et al., J. Biol. Chem. 116:*827-832 (1992). Initially, host cells expressing CCR11 are loaded with ^{3}H-inositol for 24 hours. Test compounds, *i.e.*, potential ligands, are then added to the cells and incubated at 37°C for fifteen minutes. The cells are then exposed to 20 mM formic acid to solubilize and extract hydrolyzed metabolites of phosphoinositol metabolism, *i.e.*, the products of phospholipase C-mediated hydrolysis. The extract is subjected to anion exchange chromatography using an AG1X8 anion exchange column (formate form). Inositol phosphates are eluted with 2 M ammonium formate/0.1 M formic acid and the ^{3}H associated wit the compounds is determined using liquid scintillation spectrophotometry.

The phospholipase C assay can also be used to identify modulators of chemokine receptor activity. The aforementioned assay is performed as described, but with the addition of a potential modulator. Elevated levels of detectable label would indicate the modulator is a stimulator; depressed levels of the label would indicate the modulator is an inhibitor of CCR11 activity.

VI. [^{3}H]Arachidonic Acid Release

The activation of GPCR's also has been observed to potentiate arachidonic acid release in cells, providing yet another useful assay for modulators of GPCR activity. See, *e.g.*, Kanterman *et al., Molecular Pharmacology, 39:*364-9 (1991). In this assay, an increase in [^{3}H] arachidonic acid in the presence of a putative modulator indicates stimulation of CCR11. On the other hand, a decrease in [^{3}H] arachidonic acid in the presence of a putative modulator indicates inhibition of CCR11.

For example, transfected cells are incubated with [^{3}H] arachidonic acid (0.02 μCi/well) to isotopic equilibrium (18-24 hours). Before the addition of candidate modulators, the cells are washed twice with 0.5 ml of serum-free Eagle's NO: 2 medium supplemented with 0.2% fatty acid-free BSA. The BSA is used to trap free arachidonic acid in the extracellular medium. The candidate modulators are added in a final volume of 0.5 ml, and the reaction is allowed to proceed for 15 minutes at 37°C. Buffer alone and mock transfected cells are used as controls. The reaction is stopped by removal of the incubator medium, which is then centrifuged at 12,000 x g for one minute to remove nonadherent cells. Four hundred microliters of the supernatant is removed and released [^{3}H] arachidonic acid is measured with a liquid scintillation counter.

VII. Extracellular Acidification Rate

In yet another assay, the effects of putative modulators of CCR11 activity are assayed by monitoring extracellular changes in pH induced by the putative modulators. See, *e.g.*, Dunlop *et al., Journal of Pharmacological and Toxicological Methods, 40(1):*47-55 (1998).

Extracellular acidification rates are measured using a Cytosensor microphysiometer (Molecular Devices Corp.). The capsule cups are loaded into the sensor chambers of the microphysiometer and the chambers are perfused with running buffer (bicarbonate free αMEM supplemented with 4 mM 1-glutamine, 10 units/ml penicillin, 10 μg/ml streptomycin, 26 mM NaCl) at a flow rate of 100 μl/min. Potential modulators are diluted into the running buffer and perfused through a second

fluid path. During each 60 second pump cycle, the pump is run for 38 seconds and is off for the remaining 22 seconds. The pH of the running buffer in the sensor chamber is recorded during the cycle from 43-58 seconds, and the pump is re-started at 60 seconds to start the next cycle. The rate of acidification of the running buffer during the recording time is calculated by the Cytosoft program. Changes in the rates of acidification are calculated by subtracting the baseline value (the average of 4 rate measurements immediately before addition of modulator candidates) from the highest rate measurement obtained after addition of a modulator candidate. The selected instrument detects 61 mV/pH unit.

Modulators that stimulate receptors result in an increase in the rate of extracellular acidification as compared to the rate in the absence of the modulator. This response is blocked by modulators which inhibit the receptor.

VIII. Chemotaxis-Based Cell Selection Assay

The chemotaxis-based assay described in detail in Example 4 above can also be used to identify ligands and modulators of CCR11.

IX. Competition Binding Assay

Whole cells expressing CCR11 on their cell surface, or isolated membranes from such cells are incubated with varying amounts of test compounds, either individually or in combination, in the presence of a defined amount of iodinated chemokine that is a ligand for CCR11 (*e.g.*, ELC, SLC, or TECK). The mixture is incubated at room temperature in a binding buffer containing 50 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $Mg2Cl_2$, 0.1% bovine serum albumin. After 1 hour, the mixture is filtered through a 96-well filter plate (MAFC N0B, Millipore) that has been treated with 0.5% polyethyleneimine for 1 hour at room temperature and washed with several volumes of wash buffer (same as binding buffer but with the addition of 0.5 M NaCl) in order to remove labeled ligand that is not specifically bound to the cell membranes. After the filter plate has dried, scintillant is added to each well and the plate is counted in a Wallac MicroBeta scintillation counter to determine the amount of labeled ligand retained in each well. If the test compound does not interact with the

receptor, then a maximum amount of radioligand will be bound. If the test compound binds competitively to the receptor, a reduced amount of radioligand will be retained in the well. The strength of the binding interaction is inversely proportional to the amount of radioligand retained in each well.

This assay can be run as a high throughput screen for agonists, antagonist, or modulators of CCR11 binding. It can also be used to determine the IC_{50} of a single test compound or ligand by testing each point in triplicate over a wide range of concentrations (usually over 6 to 8 orders of magnitude).

EXAMPLE 8

Identification of Chemokine Ligands of the Bonzo Receptor

A. Cloning and Expression of Bonzo

Several groups have independently identified an "orphan" G-protein coupled receptor, alternatively designated Bonzo [Deng *et al.*, *Nature, 338*: 296-300 (1997)], STRL33 [Liao *et al.*, *J Exp Med, 185*: 2015-23 (1997)], or TYMSTR [Loetscher *et al.*, *Curr. Biol., 7*: 652-60 (1997)]. The deduced protein sequence of Bonzo (SEQ ID NO: 18) was most similar to known chemokine receptors, with greatest homology to CCR7 (about 37% identity). The Bonzo gene was mapped to human chromosome 3, where many CC chemokine receptors are located. Bonzo mRNA was found to be expressed at high levels in lymphoid tissues such as spleen, lymph node, and thymus. The mRNA levels could be induced in peripheral blood leukocytes, or in purified T cells, using IL-2. Bonzo also acted as an HIV co-receptor, similar to some other chemokine receptors. However, Bonzo is unique in that it can interact with both M-tropic and T-tropic strains of HIV.

Because of Bonzo's similarity to known chemokine receptors, we and others have searched for a chemokine ligand for Bonzo by examining responses of Bonzo- expressing cells to various chemokines. Liao *et al.*, *supra*, and Loetscher *et al.*, *supra*, each reported that they tested many available chemokines, but none were found to interact with Bonzo. We selected several CC chemokines that had not been previously examined, and found one chemokine, known as SLC (Secondary Lymphoid-tissue Chemokine) [Nagira *et al.*, *J Biol Chem, 272*: 19518-24 (1997)] or

6Ckine [Hedrick and Zlotnik, *J Immunol, 159*: 1589-93 (1997)], which could induce chemotaxis of Bonzo-expressing cells.

To conduct our search for a ligand for Bonzo, we first isolated the coding region of the Bonzo gene (SEQ ID NO: 17) and prepared it for expression in mammalian tissue culture cells that could be used in functional chemokine assays. The entire coding region of the Bonzo gene was amplified from human genomic DNA using specific primers:

Bonzo 5' 5'-GCTAAGCTTCATCAGAACAGACACCATG (SEQ ID NO: 19)

Bonzo 3' 5'-GCTTCTAGAAACCCTGGCAAGGCCTA (SEQ ID NO: 20)

and inserted into the pNEF6 expression plasmid using the *Hin*dIII and *Xba*I cloning sites. The underlined portions of the Bonzo 5' and Bonzo 3' primers denote the *Hin*dIII and *Xba*I cleavage sites, respectively. Description of the pNEF6 vector may be found in US Patent No. 5,888,809..

The pNEF6-Bonzo construct was electroporated into mouse L1.2 pre-B cells for expression. Stably transfected cells were selected in 800 µg/mL G418 for 18 days. Live cells were then separated from dead cells on a Histopaque® gradient and returned to culture in G418-containing medium.

B. Primary Migration Assay

L1.2/Bonzo cells prepared as described above were tested for responses to various chemokines in a chemotaxis assay. Four chemokines were chosen that had not previously been tested for interactions with Bonzo: SLC, TECK, MIP-1δ/HCC-2, and HCC-4. Sterile Transwell® (3 µm pore; Costar) chemotaxis chambers were assembled with 5 x 10^5 cells in the upper wells and 70 ng/mL of each chemokine in the lower wells. After incubation at 37°C for 6 hours, the lower wells were examined under a microscope to determine if any cells had migrated toward any of the chemokines. Only the SLC wells contained a significant number of migrated L1.2/Bonzo cells. The other chemokine wells contained no migrated L1.2/Bonzo cells. Non-transfected L1.2 cells did not migrate toward SLC.

The L1.2/Bonzo cells that had migrated toward SLC were collected in a sterile hood and expanded. These cells were tested in chemotaxis assays toward

SLC, at several concentrations up to 1000 ng/mL. In one experiment, we could no longer see cell responsiveness to SLC. In a second experiment, (where L1.2 cells were again transfected with pNEF6-Bonzo and collected after migration toward SLC) we did observe dose-dependent migration of the L1.2/Bonzo cells toward SLC. There was some day to day variability in the absolute numbers of cells that migrated toward SLC. The L1.2/Bonzo cells that migrated toward SLC were cloned by limiting dilution and individual clones have been identified that respond to SLC at or above the levels of the originally isolated population of cells.

C. Chemokine Screening Assay

L1.2/Bonzo transfectants were originally selected by migration toward SLC, one of four chemokines that hadn't been previously tested as possible ligands for Bonzo. The chemokine-selected cells were cloned by limiting dilution and three clones that continued to respond well to SLC were examined further. It was noted again that the responsiveness of the clones decreased over a matter of weeks in culture. Accordingly, each of the clones was cloned again by limiting dilution in an effort to isolate a more stable cell line. The sub-clones were expanded and tested for responsiveness to a panel of chemokines at 20 ng/mL and 200 ng/mL in chemotaxis assays: SLC, ELC, MIP-1α, MIP-1β, RANTES, MCP-1, MCP-2, MCP-3, MCP-4, Eotaxin, Eotaxin-2, Eotaxin-3, MDC, TARC, LARC, I-309, TECK, PARC, HCC-1, HCC-2, HCC-3, Fractalkine and Lymphotactin. The L1.2/Bonzo cells responded not only to SLC but also to ELC, MCP-4, MCP-2, and MCP-1, in decreasing order of responsiveness. All of these chemokines (other than SLC) had been tested by others as possible ligands of Bonzo, as described in previous publications [Liao et al., *J Exp Med, 185*: 2015-23 (1997); Loetscher *et al., Curr Biol, 7*: 652-60 (1997)], and found to be negative. A principal factor contributing to this observed success using the method of the invention is that the responding cells (*i.e.*, those that had been selected) were isolated from the non-responding cells. In effect, the panel of chemokines was tested using a substantially more homogeneous population of receptor-expressing cells. Another factor that may have contributed to this result is the method by which the transfectants were made. Moreover, we were careful to use cells that had not been

in culture very long, as we have seen responsiveness decrease over time in culture. It is clear, however, that the chemoselection method of the invention greatly increases the sensitivity (alternatively, increases or amplifies the signal-to-noise ratio) of the screening assay, enabling the artisan to more readily identify ligand/receptor pairings that would otherwise go undetected.

More thorough chemotaxis response curves were generated using L1.2/Bonzo cells and all of the identified agonist chemokines. In this assay, MCP-4 and SLC attracted the same number of L1.2/Bonzo cells. However, MCP-4 seems to be the more potent agonist, as peak chemotaxis toward MCP-4 occurred at 10 ng/mL, whereas peak chemotaxis toward SLC occurred at 100 ng/mL. Chemotaxis toward ELC and MCP-2 also peaked at 10 ng/mL, but attracted many fewer cells.

From our analysis of L1.2-transfected cells, including the Northern blot study referred to in Example 4, we have concluded that chemotaxis selection as described herein has the potential to select for rare populations of cells which express receptors which are distinct from the receptor which was originally used for transfection. For example, the chemokine receptor CCR2 interacts with chemokines within the MCP family. L1.2 cells do not normally express CCR2 or migrate in response to MCP-4 or other members of the MCP family. However, following four rounds of chemoselection using MCP-1 as the agonist, CCR2 expression could be readily detected in the selected L1.2 cells. Consistent with this observation, these selected cells bound iodinated MCP-1, and this binding could be competed using either unlabelled MCP-1 or other members of the MCP family. This pattern of binding was also found if murine CCR2 was cloned and directly expressed in L1.2 cells.

Interestingly, L1.2 cells transfected with the chemokine receptor, BONZO (Example 8) were chemoselected using the chemokine SLC. The SLC "selected" cells, but not the parental L1.2 cells, were found to express CCR7, a previously identified receptor for SLC.

These findings have implications for the utility of the process of chemoselection to identify ligands for orphan GPCRs. Following identification of a

ligand receptor pair using the process of chemoselection, it is important to verify that the transfected receptor is expressed at high levels in these chemoselected cells (either by northern blotting or by antibody staining). Such cells preferably are analyzed for previously known receptors (if any) of the ligands identified using chemoselected cells.

The ability of chemoselection to identify rare populations of cells which express a functional receptor allows for another potential use of this technique. Putative chemoattractants can be screened against heterogenous populations of potentially responsive cells (e.g., peripheral blood mononuclear cells or thymocytes). Responsive cells can be selected for by single or repeated rounds of chemoselection. Using standard cDNA substractive hybridization technologies in which mRNAs selectively expressed by the chemoselected cells are isolated, receptor(s) for this chemoattractant can be identified. This represents yet another aspect of the invention.

All publications and patent documents mentioned hereinabove are incorporated herein by reference for all that they disclose.

The present invention is not to be limited in scope by the exemplified embodiments which are intended only as illustrations of single aspects of the invention. Indeed, numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the preferred embodiments described herein. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 14 and 25, line 21 and 22.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLTECTION (ATCC)

Address of depositary institution *(including postal code and country)*
10801 University Blvd.
Manassas, VA 20110-2209
United States of America

Date of deposit	Accession Number
2000.03.02 (2000 MAR 02)	PTA-1439

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European patent or a patent in Norway is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or the corresponding information concerningh the patent in Norrway or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) and the corresponding regulations in Norway).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

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WE CLAIM:

1. An isolated polynucleotide comprising a nucleotide sequence that encodes a CCR11 polypeptide selected from the group consisting of:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1;

(b) a polynucleotide encoding the amino acid sequence of SEQ ID NO: 2; and

(c) a polynucleotide that hybridizes to the complement of the polynucleotide of (a) under moderately stringent conditions.

2. The polynucleotide of claim 1 which is a DNA.

3. The DNA of claim 2 which is a wholly or partially chemically synthesized DNA molecule.

4. The DNA of claim 2 which is a cDNA.

5. The DNA of claim 4 comprising the nucleotide sequence set out in SEQ ID NO: 1.

6. The DNA of claim 2 which is a genomic DNA.

7. The DNA of claim 6 comprising the insert of plasmid H67224 (ATCC Accession No. PTA-1439).

8. An antisense polynucleotide which specifically hybridizes with the polynucleotide of claim 6.

9. An expression vector comprising a polynucleotide according to any one of claims 2-8.

10. A host cell transformed or transfected with a polynucleotide according to any one of claims 2-8 or a vector according to claim 9.

11. The host cell of claim 10 wherein the polynucleotide is in operative association with a regulatory sequence that controls expression of the polynucleotide in the host cell.

12. A purified and isolated CCR11 polypeptide comprising an amino acid sequence encoded by a polynucleotide according to any one of claims 1-8, or a fragment thereof comprising an epitope specific to the CCR11 polypeptide.

13. A purified and isolated CCR11 polypeptide comprising:

(a) the amino acid sequence of SEQ ID NO: 2; or

(b) a fragment of (a) wherein said fragment comprises at least one epitope specific to said CCR11 polypeptide.

14. The purified and isolated polypeptide of claim 12 or 13 comprising at least one extracellular domain of CCR11.

15. The purified and isolated polypeptide of claim 12 or 13 comprising the N-terminal extracellular domain of CCR11.

16. A purified and isolated polypeptide according to claim 12 or 13 comprising a CCR11 fragment selected from the group consisting of the N-terminal extracellular domain of CCR11, a transmembrane domain of CCR11, an extracellular loop connecting two transmembrane domains of CCR11, an intracellular loop connecting two transmembrane domains of CCR11, the C-terminal cytoplasmic domain of CCR11, and fusions thereof.

17. A purified and isolated polypeptide according to claim 12 or 13 comprising a full length CCR11 polypeptide.

18. A method for producing a CCR11 polypeptide or polypeptide fragment comprising the steps of:

(a) growing the host cell of claim 10 or 11 under conditions appropriate for expression of the polypeptide; and

(b) isolating the polypeptide from the host cell or its growth medium.

19. A composition comprising the polypeptide according to any one of claims 12-17 and a pharmaceutically acceptable carrier.

20. An antibody specifically immunoreactive with a polypeptide according to any one of claims 12-17.

21. The antibody of claim 20 which is a monoclonal antibody.

22. A cell line which secretes the antibody of claim 19.

23. The antibody of claim 20 which is a humanized antibody.

24. The antibody of claim 20 which is a chimeric antibody.

25. The antibody of claim 20 which is a single chain antibody.

26. The antibody of claim 20 which is a human antibody.

27. The antibody of claim 20 which is a bispecific antibody.

28. The antibody of claim 20 which is a CDR-grafted. antibody.

29. An anti-idiotype antibody specifically immunoreactive with the antibody of claim 21.

30. A chimeric polypeptide comprising a polypeptide of any one of claims 12-17 fused to one or more domains of an Ig superfamily protein.

31. The chimeric polypeptide of claim 29 wherein said fusion is to at least one portion of the constant region of a human immunoglobulin.

32. A method of screening cells modified to express a cell surface protein, wherein said protein is not known to mediate chemotaxis, comprising:

(a) contacting the cells with a test compound; and

(b) detecting chemotactic migration by the cells in response to the test compound,

whereby chemotactic migration by the cells indicates that the cells express the protein as a functional receptor that mediates chemotaxis in response to the test compound and/or that the test compound is a chemoattractant ligand for the protein.

33. A method according to Claim 32, wherein the protein is a G-protein coupled receptor.

34. A method according to Claim 32 or 33, wherein the test compound is known to mediate chemotaxis through a known chemoattractant receptor.

35. A method according to Claim 32 or 33, wherein the test compound is not known to mediate chemotaxis through a known chemoattractant receptor.

36. A method according to Claim 32 or 33, wherein the test compound is selected from the group consisting of chemokines, chemotactic lipids, chemotactic peptides, and functional analogs thereof.

37. A method according to any one of claims 32-36, further comprising steps of:

(c) isolating a cell that exhibits chemotactic migration in step (b); and

(d) culturing the cell in a medium that supports growth and proliferation of the cell;

whereby the method provides a clonal population of cells expressing the protein as a functional receptor that mediates chemotaxis in response to the test compound.

38. A cell culture comprising cells that have been isolated and cultured according to Claim 37.

39. A cell culture according to claim 38 that expresses increased amounts of the protein compared to the cells of step (a).

40. A method of screening cells expressing a chemoattractant receptor, comprising:

(a) contacting the cells with a test compound, wherein said test compound is not known to elicit chemotaxis through the receptor; and

(b) detecting chemotactic migration by the cells in response to the test compound,

whereby chemotactic migration by the cells indicates that the cells express the receptor as a functional receptor that mediates chemotaxis in response to the test compound and/or that the test compound is a chemoattractant ligand for the receptor.

41. A method according to Claim 40, wherein the receptor is a G-protein coupled receptor.

42. A method according to Claim 40 or 41, wherein the receptor is not known to mediate chemotaxis in response to a known chemoattractant.

43. A method according to Claim 40 or 41, wherein the receptor is known to mediate chemotaxis in response to a known chemoattractant.

44. A method according to any one of claims 40-43, wherein the chemoattractant is selected from the group consisting of chemokines, chemotactic lipids, and chemotactic peptides, and functional analogs thereof.

45. A method according to any one of claims 40-43, further comprising steps of:

(c) isolating a cell that exhibits chemotactic migration in step (b); and

(d) culturing the cell in a medium that supports growth and proliferation of the cell;

whereby the method provides a clonal population of cells expressing the protein as a functional receptor that mediates chemotaxis in response to the test compound.

46. A cell culture comprising cells that have been cultured according to Claim 45.

47. A method of isolating cells expressing a chemoattractant receptor of interest, comprising steps of:

(a) providing a reservoir comprising a cell-permeable membrane separating a cell chamber and a ligand chamber in the reservoir, wherein the cell chamber contains receptor-expressing cells in a cell medium;

(b) adding a ligand composition to the ligand chamber of the reservoir and permitting the ligand composition to contact the cell-permeable membrane;

(c) incubating the cells for a time sufficient to allow chemotactic migration of cells that are chemotactic to the composition across the cell-permeable membrane; and

(d) collecting cells that migrated across the cell-permeable membrane during the incubating step, thereby providing a cell population enriched for cells that express a functional receptor for a chemoattractant that is present in the composition.

47. A method of isolating cells expressing a chemoattractant receptor of interest, comprising steps:of:

(a) exposing receptor-expressing cells to a concentration gradient of a chemotactic agent;

(b) incubating the cells for a time sufficient to allow chemotactic migration of cells toward increased concentrations of the chemotactic agent; and

(c) collecting cells that migrated to provide a cell population enriched for cells that express a functional receptor for a chemoattractant that is present in the composition.

48. A method according to claim 47, comprising steps of:

(d) culturing the cells collected in step (c); and

(e) repeating steps (a)-(c) with the cells cultured according to step (d).

49. A cultured cell line comprising cells prepared according to the method of claim 46, 47, or 48.